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ASSESSMENT OF BACTERIAL CONTAMINATION OF
RETRIEVED MUSCULOSKELETAL ALLOGRAFT TISSUE

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THESIS SUBMITTED FOR THE DEGREE OF MASTER OF
PHILOSOPHY

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Abstract

Tissues retrieved from cadavers are increasingly banked and used for transplantation but few bacteriological standards for the safety of the tissues have been established. This study sets out i) to investigate the sources of contamination of the cadaveric tissues, ii) to determine the optimal method for sampling of tissue for bacteria and iii) to assess the use of ethanol as a general purpose sterilant for contaminated tissues.

The sources of contamination were investigated by attempting to match the DNA fingerprints of bacteria recovered from tissue post retrieval with isolates recovered from donor's skin, mortuary air and retrieval staff clothes. Isolates were identified to species level and DNA fingerprints were determined by random amplified polymorphic DNA (RAPD) profiling.

Contaminated tissues were found in 18 of 20 retrievals. Staphylococcal species, particularly coagulase negative species were among the most prevalent bacteria recovered from tissues. *Staphylococcus epidermidis* was present in 70% of the 20 retrievals, followed by *Micrococcus* spp. (50%), and *S. capitis* (45%). Other organisms were relatively uncommon, although *Escherichia coli* was recovered from 5 donors.

RAPD profiles showed that 16/35 (46%) of tissue and donor isolates of the same species from each retrieval were indistinguishable from each other and the corresponding rate of RAPD profiles of isolates from staff and tissue was 31%. There was no significant difference between the number of matches of isolates from donor and staff, and retrieved tissue isolates ($P>0.05$). Both staff and donor were the most common sources for contamination of cadaveric tissue during retrieval and to a lesser degree, the circulating air in mortuaries (20%).

Experiments showed that culturing of small aliquots of wash solutions was insensitive for the detection of bacteria. Culture of a filtered wash detected both high and low levels of contamination but was not reproducible. Swabbing of the tissue surface with a moist swab also gave variable results but did allow the growth of various bacteria following enrichment in liquid media.

Exposure to 70% ethanol for 60 min proved to be an effective sterilant of contaminated tendons, but it was necessary to remove as much muscle tissue as possible from the tissue before the treatment. Pre-washing of tissue in a mild detergent improved the effectiveness of alcohol treatment.

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Chapter 1

Introduction

Tissue banking

The retrieval, processing, storage and issue of human tissues for use in the treatment of other humans is relatively new and is generically referred to as tissue banking. A modern tissue bank may issue a wide variety of tissues to a range of medical disciplines e.g. whole bones, bone chips, tendons and ligaments, heart valves, skin, amniotic membrane, corneas, peripheral blood stem cells and umbilical cord blood, among others. In the UK, tissue banks operate to tightly controlled accredited standards to ensure the safety and function of the product and are subject to regulators appointed by the Minister of Health.

1.2 History

The age of banking tissue is relatively new, but tissue transplantation has a long history. The use of bone for transplantation was first described as early as the 17th century by a Dutch surgeon Job Van Meekeren, who grafted bone from a dog's skull to repair a defect in a soldier's cranium, which successfully healed (De Boer 1988). However, the pioneering clinical application of human allograft bone was successfully performed in 1881 by Macewen (for earlier references, see De Boer 1988) who transplanted bone taken from a child into another child. Later, the use of cadaveric bone for skeletal defects was reported in 1908 by Lexer, who made a breakthrough in the transplantation of skeletal allografts. He successfully performed 23 whole and 11 hemi-joint transplants about the knee using skeletal allografts obtained at amputation or from cadavers (De Boer 1988, Mankin *et al.* 1983). Lexer subsequently published a long-term follow-up of his study in 1925 (Mankin *et al.* 1996). Nevertheless, it was not until the mid 1900s, that the practice of banking bone became widespread. Hyatt (1976) set about developing the basic essentials for banking tissues, and established the first tissue bank in 1949 at the USA Navy Hospital, Bethesda, Maryland. The expansion of tissue

banking, however, began during the 1970s mainly in the US and Canada, and since then, a number of banks have been established worldwide, including the UK and other European countries (Villar 1991). Some tissue banks are involved in the procurement, processing and distribution of multiple types of tissue, whereas others are limited to a single tissue such as bone, skin, heart valves or corneas.

1.3 Allograft and autograft tissue transplantation

Tissue taken from an individual (donor) for the use in another individual (recipient) is termed an allograft whereas tissue from an individual is excised from one area and then grafted back into another area of the same individual is termed as autograft. Allografts are widely used in clinical practice for the treatment of disease. Dodd *et al.* (1988) compared the use of femoral head allografts versus autograft bone for the correction of idiopathic scoliosis in two groups of patients. They found a marked reduction in operative time and blood loss in the patients who received donor bone and a much lower incidence of post-operative problems. Allograft transplantation does not require the sacrifice of normal structures (Mankin *et al.* 1983) during joint reconstruction, and there is practically no limit to size and quantity of the allograft tissue used. The other benefits of using allografts are decreased surgical time and anaesthesia and so reduces morbidity and sometimes mortality (Scarborough 1992, Morgan *et al.* 1993, Misch & Deitsh 1993). Allograft bone is also larger in quantity and can be trimmed more easily to fit. In contrast, autograft transplanted bone requires longer and more complicated surgery and is associated with increased donor site morbidity.

There are some disadvantages with the use of allografts, one of which is that they undergo biological changes that are qualitatively similar, but they are usually quantitatively and temporally inferior to autografts (Friedlaender 1987). A significant attendant risk is disease transmission from donor to recipient which can lead to serious complications and death (Lord *et al.* 1988, Tomford *et al.* 1990, Mankin *et al.* 1996, Tomford 1995, 2000), such as a death of a man from *Clostridium sordellii* sepsis after receiving a cadaveric musculoskeletal allograft from a tissue bank (Kainer *et al.* 2004). Indeed, numerous incidents of person-to-person disease transmission have been reported with the use of different tissues (Gottesdiener 1989, Kakaiya *et al.* 1991, Eastlund 1995), and so avoidance of disease is paramount. Table 1 gives examples of infectious complications resulting from the use of cadaver or live donor tissues. However, despite the disadvantages, the convenience of using allograft tissue has popularised and stimulated tremendous growth in the tissue banking industry.

Table 1. Infectious diseases associated with source of tissue (Gottesdinener 1989, Steckler and Eastlund 1991, Eastlund 1995, Kainer *et al.* 2004, Gamma *et al.* 2005).

Tissue	Donor	Infectious disease
Tendon	Cadaver	<i>Clostridium sordellii</i> infection
Bone	Cadaver	Bacterial infection
Cornea	Cadaver	Rabies, Creutzfeldt-Jacob disease (CJD), bacterial & fungal infection
Dura	Cadaver	CJD
Heart-valve	Cadaver	CJD
Skin	Cadaver	Tuberculosis (TB)
Bone	Living	Bacterial infection, Human
Bone marrow	Living	CMV, Epstein-Barr virus, HIV-1, hepatitis, tuberculosis
Heart valve	Living	Candida infection,

1.4 Types of tissue banks

Tissue transplantation and banking of tissue has grown rapidly in many countries. The first organised tissue bank in the UK was established in the 1950s which banked only corneas. Today tissue banks vary in size, scope and service area, ranging from a few hospital based units and independent tissue banks, to regional or national based centres.

1.4.1 Hospital tissue banks

Hospital tissue banks are those that are attached to specialist units such as a burns centre or orthopaedic facility and generally specialise only in one type of tissue, such as skin or bone. The bone bank units have household freezers to store small quantities of sterile allografts removed in the course of routine surgical procedures and which are used for subsequent transplantation needs limited to that institution. Bone banks have become established within the hospital environment because of the relative ease with which these tissues can be banked. The surgeons have full knowledge of the donors' and recipients' medical history and are able to have control and easy access to the stored tissue (Tomford *et al.* 1986). There are however, disadvantages to hospital based tissue banks such as the limitations of the range of tissues that can be provided and a restricted number of surgeons using these tissue within the institution. It may also be the case that some surgeons have encouraged their patients to donate tissues.

1.4.2 National organisations

The alternative to hospital based tissue banks are community-wide regional or national organisations such as the National Blood Service (NBS). The National Blood Services in the UK (The English National Blood Service, NBS, and the Scottish National Blood Transfusion Service, SNBTS) in common with other countries have extended their traditional role of supplying blood products to include organ and tissue banking. The NBS first began to bank live donor femoral heads and knee trimmings at the request of

local orthopaedic surgeons (Warwick *et al.* 1996). Today the NBS operates living and cadaveric donor programmes and banks bones, tendons, skin, heart valves, peripheral blood stem cells and umbilical cord blood. The London Southeast Tissue Services (LSTS) is a constituent of the NBS and currently banks various types of tissues. At present, there are 33 tissue banks registered with the British Association of Tissue Banking (BATB) in the UK, and of these 8 are administered through the NBS (www.batb.org.uk).

1.4.2.1 An ideal partnership between tissue banks and the National Blood Service

The British Orthopaedic Association recommended that bone banking should be organised in a similar fashion to blood banking in order to ensure similar levels of safety and security. The NBS have readily available capabilities and experience with an established infrastructure. It is compliant with Good Manufacturing Practice (GMP). GMP, also known as cGMP, is a set of regulations set out by the Food and Drug Administration (FDA) to help ensure that various products intended for human consumption and use are safe and effective.

GMP regulations mandate a quality-related methodology to manufacturing, enabling businesses to minimize product contamination, mis-labelling and other errors. This protects the consumer from purchasing a product that may be defective or even dangerous.

Most of the GMP regulations primarily address issues such as sanitation, process validation, equipment and document traceability, and personnel qualification. Failure of firms to comply with GMP regulations can result in product recall or seizure, fines and prison (www.gmpguide.com).

This places the NBS in an ideal position to provide tissue banking services safely and cost effectively. As a result, the numbers of tissue banks have grown within the NBS in recognition of the benefits of applying the principle of blood banking to other human tissues. Such benefits are carefully controlled and include donor recruitment, eligibility determination and donor testing (Steckler & Eastlund 1991). Since the development of quality standards by international medical associations (American Association of Tissue Banking, AATB, European Association of Tissue Banking (EATB)) and national specialist societies (British BATB and Spanish Association of Tissue Banking, SATB), a number of local hospitals banks found difficulties in fulfilling the requirements demanded by the larger bodies, not only for training personnel and technical procedures, but also with financial viability (von Verson 1999). In the UK, the voluntary Code of Practice (COP) for tissue banking was instigated by the Department of Health (DH) in 2001 with the recommendations that NHS hospitals should only obtain tissues from accredited banks (initially by 2003, but the deadline was extended due to difficulties in some banks getting re-build/cleanrooms etc in place (personel communication, Stefan Poniatowski).

The involvement of the NBS in Tissue Banking has been well recognised by different professional groups. Steckler and Eastlund (1991) suggested that tissue banking is more efficient when co-ordinated in a regional programme instead of individual hospitals or independent tissue banks. Control of tissue banks led by regional blood centres has resulted in the development and application of more stringent criteria for medical suitability of donor tissue, more consistent donor testing and bone sterility testing along with improved storage conditions. In addition, the NBS framework ensures that tissues can be traced reliably from donor to recipient. The NBS, has now merged with the UK

Transplant to form the 'NHS Blood and Transplant' since October 2005, which will ensure a safer, more reliable and efficient way of tissue banking.

1.5 Sources of allograft tissue

1.5.1 Surgical bone donation

The LSTS currently operates two main types of tissue donation programme: 1) surgical bone donation (living donors), and 2) tissue donation after death (cadaveric). During orthopaedic surgery, in which, a patient undergoes a primary joint replacement or a total knee replacement, a portion of the bone is removed and donated to the Tissue Bank with the patient's consent. During 2003 and 2004, in the UK, 1611 femoral heads were donated. Surgical bone donation therefore provides a major source of bone tissue for the community bank (Gross 1988) with femoral heads being the most commonly banked specimen. The LSTS currently retrieves surgically removed femoral heads from around 100 hospitals, and this is continuously expanding. The femoral heads and bacterial swab samples are collected and returned to the tissue bank at -80°C within 24h of retrieval. Blood samples are tested for anti-HIV 1 and 2, anti-HCV, hepatitis B surface antigen and syphilis antibodies at the NBS.

1.5.2 Tissue donation after death

In contrast to living donors, cadaveric donors are a source for a number of different types of tissue. With the consent of the next of kin, tissues such as, cornea, skin, connective tissue, heart valves, bone and tendons can be retrieved. Examples of the types of tissues used for surgical treatment from living and cadaveric donors are presented in Table 2.

Table 2. Estimated numbers of tissue allografts used annually in the USA (Steckler & Eastlund 1991, McCullough 1991).

<i>Cadaver tissue</i>	<i>Transplants</i>	<i>Indication</i>
Bone	250 000	Spinal fusion, revision of failed hip prosthesis. Fill periodontal defects; fill defects caused by malignancy or trauma
Cornea	36 000	Corneal oedema, herpetic scars,
Skin	5000	Temporary cover for third degree burns
Heart valve	2000	Replace damaged valves
Cartilage	1100	Maxillofacial reconstruction, repair damaged articular cartilage
Tendon	500	Replace injured knee ligaments
Fascia	500	Correct ptosis (drooping of upper eyelid)
Dura	500	Keratoconus (conical protrusion of cornea)
Pericardium	100	not provided
Saphenous vein	100	Coronary heart by-pass graft, leg arterial vascularisation
<i>Living donor</i>		
Bone	5000	not provided
Bone marrow	2000	Aplastic anaemia, Leukaemia

1.6. Bone allografts

Bone is the most commonly transplanted tissue after blood (Scarborough 1992) and is undertaken by orthopaedic centres world-wide (De Boer 1988). A decade ago, over 300,000 bone allografts were transplanted annually compared with about 20,000 cadaveric organ transplants in the United States (Warwick *et al.* 1996). The use of bone grafts is higher in the US compared to the UK partly due to extensive use by dental practitioners accounting for 50,000 to 100,000 vials of powdered bone allograft annually. Data from the UK and Ireland show similar trends on a smaller scale and the surgical demand for allografts in the UK will inevitably increase further.

The number of bone allografts issued nationally 2003/2004 was as follows (includes cadaveric and surgical) (data provided by Amanda Ranson, LSTS):

- Fresh frozen femoral heads - 1612
- Irradiated frozen femoral heads - 764
- Washed frozen irradiated whole femoral heads - 105
- Washed frozen irradiated half femoral heads - 74
- Washed freeze-dried irradiated whole femoral heads - 35
- Washed freeze-dried irradiated half femoral heads - 157
- Frozen ground bone - 52,815 cc
- Freeze-dried ground bone – 37550 cc
- Freeze-dried rings – 404 mm
- Freeze-dried femoral slices – 40
- Frozen struts – 3149 cm
- Freeze-dried struts – 1624 cm
- Freeze-dried cubes – 259
- Freeze-dried pegs - 39
- Freeze-dried chips – 100

Bone grafting is considered to have a 3-fold function (Prolo & Rodrigo 1985): i) it gives stability to weakened bone; ii) it stimulates osteogenesis and iii) it serves as a matrix for the bone formation which then assumes the shape and extent of the graft. The four main types of bone allografts are shown in Figs. 1 to 4.

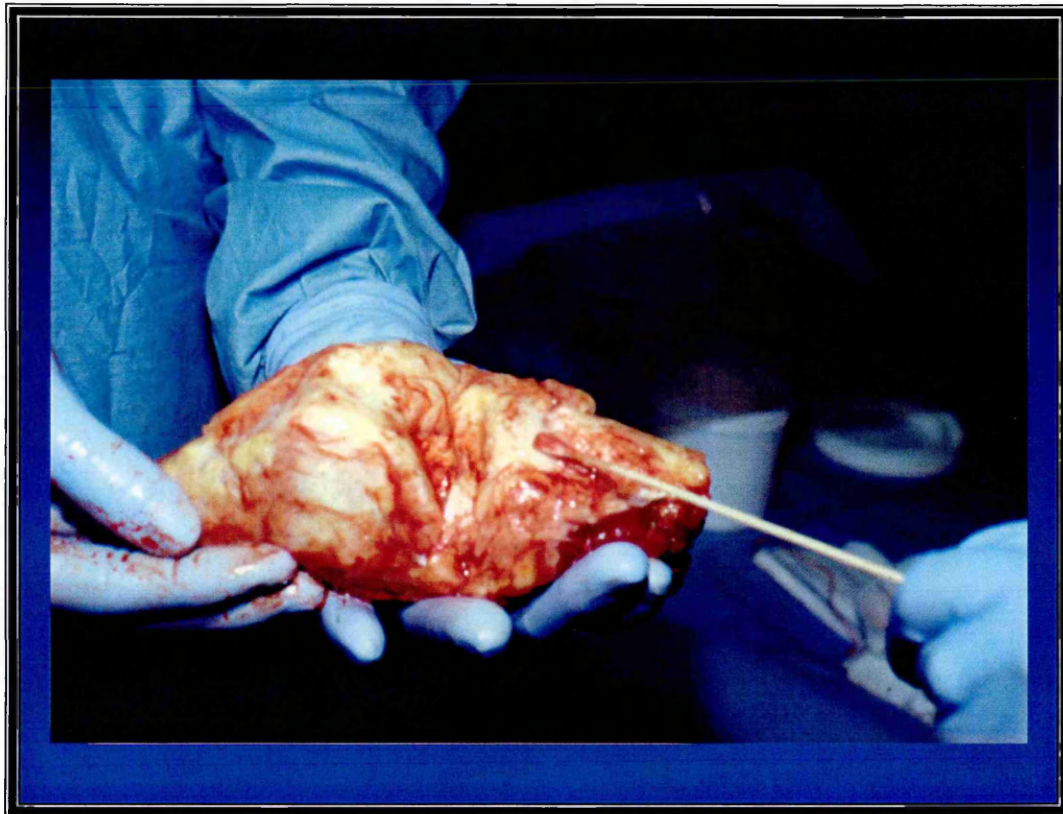


Figure 1. Fresh frozen knee allografts



Figure 2. Lyophilised and demineralised bone



Fig. 3. Reshaped irradiated Patellar and Achilles tendon

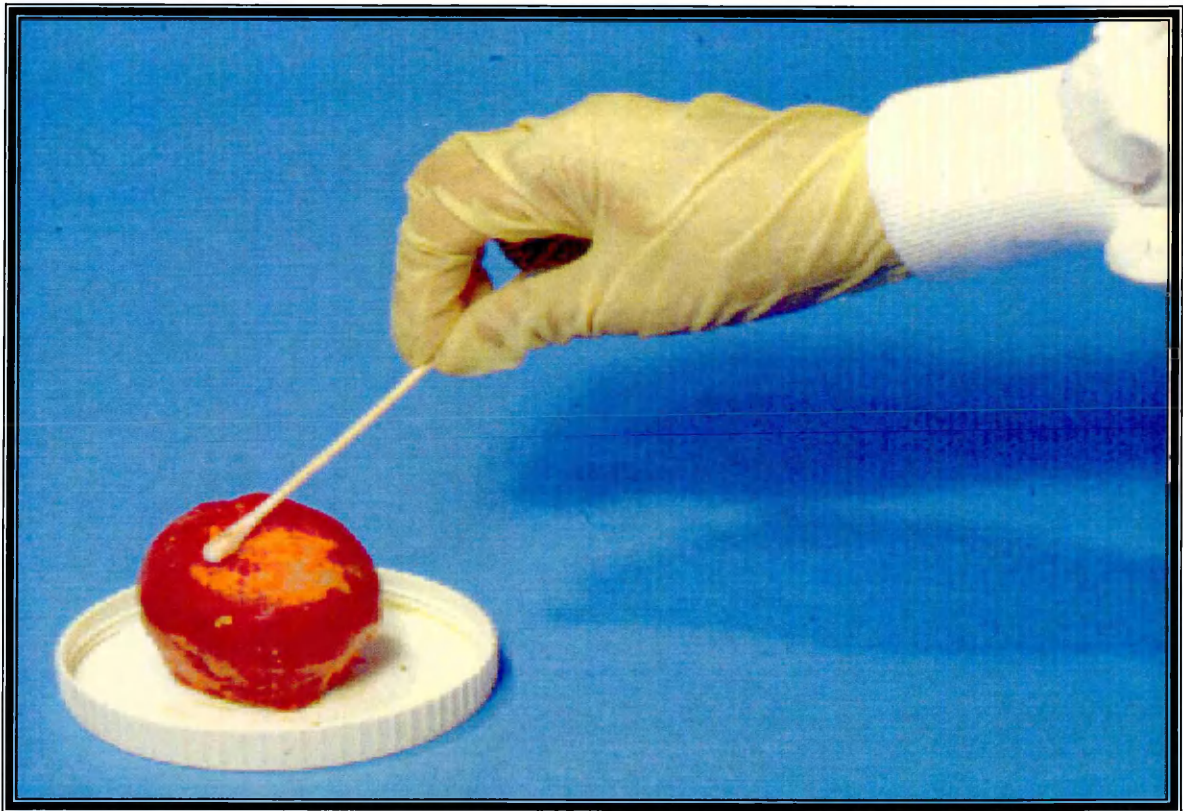


Fig 4. Fresh frozen femoral head

Bone grafts are required for a number of orthopaedic procedures, ranging from correcting spinal abnormalities, reconstruction of large skeletal defects after tumour resection or traumatic bone loss owing to diseased or damaged bone (De Boer 1988). At least half of primary bone replacements require surgical revision after time. Bone grafting is carried out to reconstitute lost bone stock in the femoral head socket and accounts for the dramatic increase in the use of artificial femoral head implant surgery over the past two decades (Gross 1988, Warwick *et al.* 1996). The replacement of lost bone stock using bone from the patient's own hip is usually insufficient for need and so the alternative is to use donated bone from the bank.

1.7. Contamination of allograft tissue

Despite careful donor selection and aseptic retrieval, contamination of retrieved tissues is common. Donor to recipient disease transmission of infection has been reported with a number of tissues (Gottesdiener 1989, Kakaiya *et al.* 1991, Eastlund 1995, Conrad *et al.* 1995), and in severe cases death of the recipient has occurred, for example, as a result of infection with clostridial species. A case of a young man who received fresh frozen osteochondral tissue, who developed abdominal pain, hypotension and died from infection due to clostridial septicemia was described by Eastlund (MMWR 2001, December). Farrington *et al.* (1994) showed that it is very difficult to avoid tissue contamination at the time of retrieval. Organisms can originate from several sources i) personnel carrying out the retrieval, ii) the donor's skin, iii) the mortuary environment or an endogenous source. The presence of organisms of low pathogenicity is mainly associated with external factors, whereas contamination with highly pathogenic organisms is usually associated with an endogenous source (Deijkers *et al.* 1997).

Examples of bacterial species considered to be of high pathogenicity (organisms that can give rise to infection at low numbers and the incubation period are short before symptoms in the patient develop) and low pathogenicity (require higher number of organisms before infection develops in a patient) are listed below:

High pathogenic organisms

Streptococcus spp.

Staphylococcus aureus

Clostridium spp.

Bacteroides spp.

Escherichia coli

Yeast,

Pseudomonas spp.

Low pathogenic organisms

Coagulase negative staphylococci

Corynebacterium spp.

Propionibacterium acnes

Bacillus spp.

Diphtheroids.

Micrococcus spp.

Sarcina

The risk of infecting the recipient can be reduced by adequate microbiological monitoring and decontamination procedures. It is vital that no major damage takes place to the tissue as a result of the decontamination process as it is known that certain methods can alter the biomechanical and biological properties of the tissue (Prolo & Rodrigo 1985).

The percentage of cadaveric tissues found contaminated nationally prior to any kind of processing in 2003/2004 was 3.28%. About 1616 components were tested and 53 were positive with bacteria. The percentage of surgical tissues found contaminated for 2003/2004 for Edgware, Cambridge, Brentwood and Oxford was 6.53% (131/2006), (NBS, Amanda Ranson).

At the LSTS, all live donor tissues that are found to be contaminated after retrieval and all cadaveric tissues are processed such as decontamination and removal of blood, marrow and lipid and the bone is fashioned to the surgeon's preferences in order to save time in the theatre which includes. Live donor tissue free of bacterial contaminants are not processed further. Most bone tissues that are processed at LSTS are freeze-dried and irradiated, however, bone can also be exposed to EtOx (ethylene oxide gas) as a sterilant. The greatest clinical demand is currently for lyophilised bone (Fig. 2). Tendons retrieved are also processed and decontaminated either by alcohol, antibiotics or irradiation.

1.7.1 The risks of disease transmission

The widening of human tissue transplantation has brought attention not only to their effectiveness and advantages over autografts, but also their drawbacks, side effects and complications such as disease transmission from donor to recipients (Eastlund 1995). As the volume of tissues banked increases, the insurance of providing safe tissue for clinical use must be stringent. The risks of disease transmission by transplanted tissues are similar to those for blood transfusion such as viral contamination bearing in mind that the level of risk is higher for tissues than for blood because tissues retrieved from a single donor may be used in multiple recipients so more stringent standards are required. Bacterial and viral infections have been transmitted by both organ and tissue transplants. (Steckler & Eastlund 1991, Eastlund 1995), and a current concern is the transmission of the agent of CJD. Unlike blood banking and solid organ transplantation, tissue banking and the transplantation of allograft tissue is considered to be a non urgent, life enhancing procedure rather than life saving (with the exception of

heart valves, skin, cord blood), such as a secondary hip revision and anterior or posterior cruciate ligament reconstruction. Multiple tissues are often retrieved from a single cadaveric donor and in practice 50 or more recipients could receive tissues from a single donor (www.advisorybodies.doh.gov.uk/acmsbtt/index), and thus the impact of any disease transmission could potentially be far greater than from a single solid organ transplantation. In such circumstances, the public support and the confidence of the surgeon using the allografts would be threatened and could damage the further development of tissue banking. It is therefore imperative that screening of donors, retrieval, processing, storage and distribution of allografts are scrupulously controlled and regulated.

1.8 Guidelines and standards

The first standards for tissue banking were established in the USA by AATB which is a scientific, not-for-profit, peer group organization founded in 1976. Its mission is to facilitate the provision of high quality transplantable human tissue in quantities sufficient to meet national needs. The standards were initially used by tissue banks in the UK and other countries such as France & Germany before they established their own tissue banking associations. The BATB was first structured in the early 1990s to bring together professionals with specialist interests in tissue banking to assist the promotion of Good Practice in Tissue Banking, to provide a forum for exchange of views among specialists and to reflect the views of the membership to appropriate external bodies, including regulatory and professional (www.batb.org.uk). The Association is recognised and consulted by the DH on matters relating to tissue banking in the UK and has relationships with the Council of Europe and the European Commission independently and through the DH.

The BATB published their own standards and technical manual outlining individual responsibilities for tissue bankers. Standards in tissue banks in the UK are controlled by responsible agencies, which are normally part of the NHS. Tissue banking standards are constantly evolving as various professional groups discuss and consider all aspects of tissue banking at annual meetings and as technology develop to reduce the risk of disease transmission and provide better preservation techniques. To ensure the smooth and safe running of a tissue bank, these Associations produce guidance and standards documents for tissue bankers. The Associations consider their 'General Standards' to be the minimum acceptable standards and through their membership, encourages tissue bankers to maintain these standards (Friedlaender & Goldberg 1989, www.batb.org.uk).

The BATB standards provide guidance on

Donor selection criteria

Donor consent

Retrieval of tissue

Donor reconstruction

Donor blood testing

Tissue processing and sterilisation

Tissue preservation

Tissue storage

Tissue release

Labelling and packaging of final products

How tissue banks screen donors, retrieve and prepare allografts impacts on the risks and benefits of these materials. The standards highlight what should be covered during donor selection or tissue processing with the main emphasis on the prevention of

disease transmission, whilst maintaining the biochemical and biomechanical properties of the allografts.

1.8.1 The Committee for the Microbiological Safety of Blood and Tissue for Transplantation.

The Committee for the Microbiological Safety of Blood and Tissue for Transplantation (CMSBTT) have published guidance on the Microbiological Safety of Human Organs, Tissues and Cells used in Transplantation (www.advisorybodies.doh.gov.uk/acmsbtt/index). They highlight the main risks associated with transplantation and give advice on minimising the transmission of disease through donor selection, serological testing, bacteriology and tissue processing. The guidelines emphasise the importance of donor selection and give examples of the types of questions to ask during the interview of the donor's medical and social life history.

1.9. Regulation and inspection

The *Guidelines for the Blood Transfusion Services* in the UK were first published in 1990 by HMSO. They were compiled by experts from the then Regional Transfusion Centres and the National Institute of Biological Standards and Control (NIBSC), and aimed to define guidelines for all materials produced by the UK National Blood Service for both therapeutic and diagnostic use. The driving force for this joint initiative, which started in 1987, was the imminent EU Directive which would bind member states to introduce product liability by July 1988. It was understood that human blood and substances derived from it would be defined as 'products' in terms of this Directive, and guidelines against which manufacturers could be inspected would be required.

The 'Red Book' contains guidelines reflecting best practice, it sets standards to be met

by the products, describes technical details of the processes involved and states the legally binding requirements introduced in 2005 under the Blood Quality and Safety Regulations, Statutory Instrument 2005 No. 50.

Since 1990, seven editions of the 'Red Book' (as the guidelines became known) have appeared. They are compiled by a group of experts many of who are from outside the blood transfusion services, now called the Joint UKBTS/NIBSC Professional Advisory Committee (JPAC) (www.transfusionguidelines.org.uk)

In the UK, COP for Tissue Banking was instigated by the DH in 2001 with the recommendation that NHS hospitals should obtain tissues only from accredited banks. The regulatory authority in the UK for COP is the MHRA-Medicine and Healthcare product Regulatory Authority (previously the Medicine Device Agency and Medicine Controls Agency merged). They inspect and license tissue banks (where volunteered) to Pharmaceutical/blood equivalent standards against the COP. All new tissue establishments will be inspected and licensed by MHRA.

Arm's Length Bodies (ALB) are key parts of the present health and social care system. As stand-alone national organisations sponsored by the DH, they work closely with the local NHS, social care services, and other ALBs to regulate the system and improve standards). An ALB was formed on the merger of NBA, Bio Products Laboratory, the International Blood Group Reference Laboratory, and UK Transplant (UKT) on October 2005 to form a new organisation, NHS Blood and Transplant (NHSBT).

The creation of NHSBT is expected to maximise the strengths of both organisations, further improve services to patients and donors and build on the excellent collaborative work already underway. The work of both the NBA and UKT is being reviewed to examine the scope for more effective and efficient operation, further modernising them in line with the wider NHS. (www.nhsbt.nhs.uk/)

The EU directive on tissue banking comes into law in early 2006 which will bring all European tissue banks under the same regulation. This will supersede the UK Code of Practice, and so establishments providing tissues for transplant anywhere in Europe will need to be inspected by law against this directive (Directive 2004/23/EC of the European Parliament and of the Council 2004). There are two technical annexes still in draft, which are likely to come into force over the coming two years (Anon). The directive covers aspects such as donor traceability, donor selection, evaluation, and consent, quality management, tissue and cell processing, and storage and distribution. The EU Directive on Tissues (2004/23/EC) will become legally binding in the UK from April 2006.

The regulatory authority set up in the UK is the Human Tissue Authority that will soon merge with the Human Fertilisation and Embryo Authority (HFEA) to form a new authority to oversee all tissues, stem cells and gamete banking under the directive as the defined competent authority. It is not yet known who they will commission to do the licensing. There is also a draft Tissue Engineering European Directive, but how this will overlap with the tissue directive is not yet clear, i.e. what constitutes 'engineering'. The Human Tissue Act 2004 (replacing the Human Tissue Act 1961) is also due to come into force in early 2006. The regulations can be viewed on www.dh.gov.uk, and it is primarily regarding donor consent.

1.10 Quality assurance

This also applies to the 'Red Book', although it is of a higher standard compared to the BATB standards, it still only gives general guidelines on tissue retrieval, cadaveric reconstruction, processing (bacteriostasis and disinfection), tissue storage, etc. There are no specific standard operating procedures, and so, based on basic guidelines, an individual tissue bank can adopt their own techniques and have their own specific standard operating procedures.

Although the BATB standards and the Red Book give an overall guide on how to manage a tissue bank, they do not give detailed instruction on the selection of donors or the specific techniques to be used for retrieval, processing and sterilising of tissues. Consequently, there is wide variation in the performance of bacteriological control of retrieving and processing of tissues and this has led to a situation where some tissue bank uses their own techniques for retrieving and processing. There is little doubt that the final outcome of the tissue and its success for transplantation differs to some extent from one tissue bank to another due to variation in the techniques used. Scientific research on these procedures is scarce and so, many tissue banks use techniques without knowing how efficient they are at performing their role. Such techniques include minimising bacterial contamination, microbiological monitoring of tissue, tissue sterilisation and preservation without affecting the biochemical or biomechanical properties of the tissue.

1.11 Microbiological quality control

The risks of transmitting bacterial infections are considered to be higher than viral infections, although the latter are more difficult to treat. Therefore, the microbiological

regulation of tissue transplantation (BATB and Department of Health Advisory Body (www.batb.org.uk, www.advisorybodies.doh.gov.uk/acmsbtt/index) concentrates on virological rather than bacteriological risks. The BATB documents and the Red Book only highlighted the importance of bacterial infections of the donor, and that tissue should not transmit difficult to treat infections. The Red Book gives information on the type of bacterial infection or bacterial tissue contamination that should be avoided for donation, however, it does not give guidelines on the interpretation of microbiological tests results of donor tissue. There are no specific protocols for minimising contamination during retrieval of tissue, bacteriological sampling, decontamination, and sterilisation of tissue. The requirement is made that the proof of the efficiency of the selected methodology is submitted within the framework of the production process (www.advisorybodies.doh.gov.uk/acmsbtt/index, www.batb.org.uk), i.e., validation of the methodology on the basis of scientific criteria appropriate to the process. Consequently, there is wide variation in the performance of bacteriological control of retrieving and processing of tissues.

Strict standards are applied to avoid disease transmission from donor to recipient: These involve taking of an accurate donor medical history, donor testing for viral diseases, aseptic retrieval and processing, and control of storage temperature of tissues. During aseptic processing, precautions are taken to minimize the introduction of new organisms and so bioburden assessment and processing of tissue is carried out using sterile equipment in a clean room with positive air pressure, inside a class II cabinet and staff wearing protective gowns, caps and face masks to avoid the introduction of bacteria. Despite these efforts to minimise contamination, incidents of contamination do occur either through retrieval or during processing. Farrington *et al.* (1996) reported contamination of bone grafts from un-sterilized de-ionizer water during processing. An

isolate closely related to *Burkholderia cepacia* was recovered from pre- irradiated bones of six donations over a period of four weeks. Their findings highlight the importance of quality control, and establishing standards, and guidelines for safe performance of bone banking.

1.11.1 Donor selection

Donor evaluation is perhaps the single most important factor in the prevention of disease transmission. Donor tissue neither improves nor becomes safer during subsequent banking procedures if the donor has an infectious disease such as HIV or vCJD (Leslie & Bottenfield, 1989). Even if terminal sterilisation is carried out on tissues that were taken from donors with HIV or vCJD, there is no guarantee that these agents will be eliminated. Careful and thorough review of the donor's medical and social history is carried out to exclude those that may be at risk of transmissible disease. In recent years, donor evaluation has evolved to include:

1. A direct interview with the donor (live donor) or the next of kin (cadaveric donors) for social and medical history.
2. GP questionnaire (medical history).
3. Pathologist questionnaire (cadaveric donor) for medical history review.
4. Physical examination of cadaveric donor by retrieval teams (evidence of IV drug abuse, recent tattoos, etc).
5. Blood testing, anti-HIV, anti-HCV, HBsAg, syphilis, and wherever possible, HIV and HCV by Polymerase Chain Reaction (PCR).
6. Donor file review by tissue bank consultant.

1.12 Aims of study

It is important that sterilisation methods are validated in accordance with precise definitions of sterilisation, and for the initial levels of "bioburden" expected to be present immediately prior to application of the sterilisation method are quantified. The application of improved and refined methodologies in accordance with defined standards will ensure improved graft performance while reducing risk to the recipient (Kearny 2005).

As there are no set bacteriological standards for the tissue banks to adhere to, the bacteriological aspects of the various procedures in tissue banking needs to be investigated, before standards can be set. The aim of the study is to:

1. Develop effective aseptic procedures for the recovery of cadaveric allografts. This can only be achieved if the major sources and routes of tissue contamination are established. This study sets out to investigate the sources of contamination of cadaver tissues by attempting to match bacteria recovered from the tissue with those found on the donors' skin, mortuary air and the staffs' clothing using molecular typing techniques. Data from this study will inform the development of effective aseptic procedures that target the main sources of contamination within a mortuary. The data should provide information on the possible breakdown in aseptic procedures such as donor's skin disinfection, and so lay the basis for the establishment of specific guidelines or effective measures to minimise contamination in tissue retrieval.

2. Develop a sampling method to reliably and efficiently represent the whole surface of the tissue that could be readily adopted as a routine sterility test at the time of surgical retrieval. The developed method will be compared with two methods for assessing bioburden on bone currently used a) a qualitative swab technique b) a semi-quantitative wash culture technique.
3. Investigate if ethanol can be used to decontaminate tendons effectively and efficiently.

Chapter 2

Establishing the sources of tissue contamination
during retrieval within mortuaries

2.1 Introduction

Musculoskeletal tissues intended for transplantation are at risk of bacterial contamination during the retrieval and processing period and the degree and type of contamination greatly influences the suitability of the tissue for subsequent surgical use. As part of the prevention of disease transmission during transplantation, the BATB standards have ruled that all tissue banks must make every effort to minimise contamination of tissue during retrieval. The standard also states that tissue should be retrieved from cadaveric donors as soon as possible after death to prevent multiplication of organisms. If it is not practical to retrieve tissue at an earlier time, the donor should be kept refrigerated prior to the retrieval and this must be performed within 48 h of death. In most cases retrieval at the LSETS is performed within 24 h of death. The standards do not specify the type of procedures tissue banks should follow within a mortuary. Consequently, individual tissue banks have adopted their own procedures and these are often similar to those used in an operating room during a surgical procedure. The principle of minimising contamination is based on performing all aspects of the retrieval as aseptically as possible.

2.1.1 Aseptic procedures

The approach and the degree of aseptic procedures for minimising bacterial contamination vary considerably from one tissue bank to another. However, the aseptic procedures used by all tissue banks are designed to protect retrieved tissue from the potential sources of contamination. The sources of tissue contamination within a mortuary are likely to be different to that of an operating room due to the differences in environment, type of work involved and working practices. Nevertheless, despite these differences, most tissue banks have not modified their aseptic procedures to accommodate the conditions within a mortuary.

Measures taken to minimise or prevent contamination of tissue are based on the knowledge that tissue can become contaminated by two routes: 1) exogenously and/or 2) endogenously. For example, exogenous contamination such as air bacteria are reduced by air filtration, staff wearing surgical suits and face masks.

During harvesting, the potential exogenous sources of contamination are considered to be:

- 1) Members of the retrieval team,
- 2) The donor's skin
- 3) Surgical instruments
- 4) Mortuary air and the general surroundings

Endogenous contamination originates from the donors' blood stream or body contents. This can be due to clinical bacteraemia and/or post mortem spread of bacteria (transmigration from body sites such as the bowel).

2.1.2 Retrieval team

Many studies have been carried out in operating theatres to assess the sources of contamination. The concept of airborne bacteria contaminating the surgical wounds were based upon correlations made between the rate of surgical infection and the level of airborne bacteria on different occasions and the type of ventilation system within the operating room. It is widely accepted that members of the surgical team are often the prime source of contamination of surgical wounds and subsequent post-operative infection (Millar *et al.* 1976, McCue *et al.* 1981, Whyte *et al.* 1982, Lidwell *et al.* 1983, Bukhari *et al.* 1993). Staff working in the operating room are also the main origin of airborne bacteria. Sciple *et al.* (1967) showed that natural walking movements released

about 10^4 skin scales per minute, of which 10% carry clusters of micro-organisms. This may settle directly on a wound or on other surfaces that are exposed to the air including instruments, the surgeon's hands and protective drapes from where they may be transferred to a wound (Hambraeus 1988, Whyte *et al.* 1992). A number of studies have been carried out to assess and design effective procedures for preventing surgical wound contamination by staff. Some of these examined preoperative measures to prevent contamination such as hand scrubbing techniques, and the contribution of sterile disposable gloves, gowns (Whyte *et al.* 1976, 1990, Scheibel *et al.* 1991, Hubble *et al.* 1996), and face-masks as barriers to transfer of microorganisms to the wound (Quesnal 1975, Davis 1991). Aseptic techniques used in operating rooms have evolved and advanced due to these studies and resulted in a variety of effective barriers to prevent dispersal of bacteria carrying particles and so reducing the incidence of wound infection (Whyte *et al.* 1990., Blomgren 1990).

2.1.3 Preventing staff contamination

Based on the possibility that staff may be potential sources of tissue contamination, the LSTS follow similar hygiene rituals to that of surgical teams. Disposable scrub suits and hair nets are worn, and hands and forearms are scrubbed with antiseptic agents. The teams also wear full non-woven sterile surgeon's gowns and double layers of sterile disposable gloves (Fig 6).

2.1.4 Donor

2.1.4.1 Donor selection

The LSTS attempts to reduce the risk of endogenous contamination of donated tissue by donor selection. Endogenous bacterial contamination can only be prevented through donor selection and not by subsequent aseptic procedures.

Donors are not recommended if 1) an infection of known aetiology is at a site distant from the donated tissue; 2) an infection is in a site in continuity with the site of the retrieved tissue; 3) the microbiology/aetiology of an infection is not certain; 4) the organism causing the infection commonly produces metastatic foci of infection in the transplanted tissue. (www.advisorybodies.doh.gov.uk/acmsbtt/index, www.batb.org.uk)

The NHS Executive guideline (www.advisorybodies.doh.gov.uk/acmsbtt/index) has given a list of infections that requires a microbiological specialist to determine the suitability of the donor. Such infections include candidiasis, brucellosis, cryptococcosis, listeriosis, meningitis and infection with methicillin resistant *Staphylococcus aureus* (MRSA).

2.1.4.2 Physical examination of the donor:

During the retrieval process, every effort is made to prevent the donor's skin flora contaminate retrieved tissue. The donor is physically examined for any signs of bacterial infection on the surface of the skin and for any signs of wounds, cuts, and abrasions which might lead to invasion of micro-organisms to the area where tissue is to be removed. For example, invasion of bacteria is likely to occur after a traumatic road traffic accident where the donor is deeply lacerated.

2.1.4.3 Donor's skin flora and disinfection

Several authors have associated patient's skin flora with surgical infection despite skin disinfection prior to surgical procedures (Hambraeus 1988, Whyte *et al.* 1992). As a standard procedure, the area of the donor's skin from which tissue is to be retrieved is disinfected with an alcohol based skin disinfectant (0.3% chlorhexidine gluconate, in 70% industrial methylated spirit (Hydrex, Adams Health Care, UK). The donor is then draped with non-woven sterile universal gowns (Johnson & Johnson Ltd), exposing only the area from which tissue is to be removed.

2.1.5 Surgical instruments:

Retrieval instruments that come in contact with the tissue are either single use disposable sterile items or are packaged and sterilised (autoclaved) between each retrieval. Other non-autoclavable instruments are sprayed with disinfectants. At the mortuary, a sterile field is created, on which sterile instruments are placed. Any used instruments are also kept on the sterile field away from the other unused instruments. However, once back at the tissue bank, any unopened disposable instruments will be used another retrieval, however any autoclavable instruments that are not used will autoclaved again before they are taken to another retrieval.

2.1.6 Rate of tissue contamination:

A survey (data collected from donor's file and database) at LSTS of 101 retrievals from cadavers showed that as many as 96% had tissues that were contaminated with bacteria. Coagulase negative staphylococci, in particular *Staphylococcus epidermidis* were among the most frequent species recovered (65%) followed by diphtheroids (32%), enterococci (9%), aerobic spore bearers (9%), and coliforms (8%). In total, 373

individual tissues were retrieved of which as many as 80% were contaminated (Table 3). On a national scale, the percentage of cadaveric tissue found contaminated was prior to any kind of processing was 3.2% in 2004 (National Bacteriology Laboratory, NBS).

Table 3. Number of individual types of tissues retrieved and the percentage found contaminated.

Tissues	Numbers Retrieved	Percentage contaminated
Femoral heads	132	81% (108)
Femoral shafts	43	79% (34)
Achilles tendons	31	94% (29)
Patella tendons	32	72% (23)
Knee joints	133	78% (104)
Semi-tendinous	2	100% (2)

A number of incidences of bacterial infection following tissue transplantation have been reported (Tomford *et al.* 1990, Kakaiya *et al.* 1991, Tomford 1995). The consequences of an infection in the recipient may lead to removal of the implant, amputation, and long term hospital stay. These reports and the results of the survey above suggest that despite compliance with aseptic procedures to minimise contamination, the rate of tissue contamination is very high suggesting that the aseptic procedures were not effective or that contamination occurred from other unrecognized sources and may have occurred within the laboratory.

2.2 Material and methods

The aim of the study was to establish the major sources of contamination of retrieved tissue within the mortuary environment. Bacteriological sampling was performed of the donor's skin, mortuary air and retrieval staff. Isolates from the source were matched for species identity and within a species for genetic relatedness. Other factors that could affect the chances of tissue contamination and type of species contaminating the tissue were also recorded, such as cause of death, the numbers of staff present during harvesting, and prior post mortem examination.

The study consisted of 5 stages

2.2.1 Stage 1: Cadaveric donor selection

The medical history, cause of death and the lifestyle of donor were recorded before a potential donor was accepted. The following information was requested of relatives and/or medical records.

2.2.1.1 Medical history enquiries-donor family

Major operations or chronic illness, tuberculosis, or blood transfusion

- treatment of cancer
- rheumatoid arthritis
- viral infection or venereal disease
- prescribed medication
- treatment with hormone injection to improve growth or fertility
- acupuncture, tattooing or body piercing
- travel outside Europe in previous year, malaria or either tropical disease
- family history of CJD

- blood donation
- any other information that may be significant

2.2.1.2 Lifestyle exclusions:

- men who have had sex with other men
- anyone who has injected themselves with non prescription drugs
- prostitutes
- people who are or who think they may be infected with HIV or HBV or HCV
- people with haemophilia who have been treated with clotting factor concentrates
- anyone who has had been sexually active in Africa within the last year (except Morocco, Algeria, Tunisia, Lybia and Egypt).
- anyone who has had sex in the previous year with anyone from the above group.

2.2.1.3 Medical history enquiries-family doctor:

- AIDS, hepatitis, tuberculosis or venereal disease
- any form of malignancy
- any autoimmune disease or disease where altered immune competence is suspected
- any disease thought to be of prion origin, e.g., CJD
- any disease of unknown aetiology (e.g., Parkinson's disease, multiple sclerosis, dementia)
- any degenerative neurological disease
- any immunisation inoculations or vaccinations in the month prior to death

- any treatment with hormone injection derived from pituitary gland
- prescribed medication
- any knowledge of lifestyle risks

2.2.1.4 Physical examination/cause of death

- cause of death
- evidence of occult malignancy
- evidence of systemic infection
- evidence of intravenous drug abuse
- evidence of CNS prion disease
- evidence of any disease of unknown aetiology including auto-immune disease

2.2.2 Stage 2: Assessment of factors that contribute to the rate of tissue contamination.

A list of factors was compiled for assessing the rate and type of tissue contamination. A total of 20 retrievals were performed and the following factors were recorded.

- time and date of death
- cause of death
- date of retrieval
- time donor was kept out of the fridge prior to retrieval
- time of retrieval
- post mortem prior to the retrieval
- time of P.M.
- name of the mortuary

- Cleanliness of the mortuary room (this was scored between 1 to 3, 1 being the cleanest (i.e., general tidiness, are the tables clean from bodily fluids, etc, is the ventilation on at all times, is the floor clean and tidy?). Although the method used to assess cleanliness was subjective, all the mortuaries were assessed by the same person. Questions on the ventilation system were asked on the day to the mortuary technician who was present at the time. It can not be confirmed that the information provided by the technician is accurate.
- any other activities carried out in the room prior to the retrieval or during the retrieval, P.M or embalming on deceased other than the donor
- type of retrieval. i.e. full (more than one type of tissue) or knees only
- length of retrieval
- number of persons present at the retrieval, including those that are not part of the retrieval team
- presence of an air extractor, and if so when functional

2.2.3 Stage 3: Bacterial sampling of potential sources and retrieved tissue

Tissue retrievals were carried out when a suitable donor was available, and this could be at any time of the day and week. Due to the nature of availability of the donor, a bank of retrieval staff was always on standby. Bacterial sampling was performed during these retrievals but further laboratory work could not always be carried out immediately. If a retrieval was done late in the evening, the bacterial samples were refrigerated until a convenient time to subculture them. However, the main concern was the viability of bacterial cells in refrigerated samples, especially if only a very small number were recovered from tissue. A controlled study was therefore carried out to determine the affects of refrigeration and sterile phosphate buffered saline (PBS pH 7.5)

containing 0.1% Triton, (Sigma, UK) on the viable counts of the bacterial cells recovered from the retrieved tissue and donors' skin. The study (appendix 7.2.2) showed that about half of the CFUs survived over a period of 48 to 72 h. Based on these results, it was decided that the bacterial samples would be subcultured on arrival at the tissue bank, and to facilitate this, a 37°C incubator was installed.

2.2.3.1 Sampling donor's skin:

Prior to skin disinfection with Hydrex containing 0.3% chlorhexidine gluconate in 70% IMS (Adams Health Care, UK), the donor's skin was sampled at the intended incision site on the left and right knee and thigh area using Williamson and Kligman's (1965) cylindrical scrub technique. A sterile rectangular plastic block (5cm x 2.5cm) with a hole in the centre of 1.6 cm in diameter was placed over the donor's skin (Fig. 5). A volume of 3 ml sterile PBS pH 7.5 containing 0.1% Triton, (Sigma, UK) was pipetted into the hole. A cotton swab was used to rub the surface of the skin within the aperture for 1 min. The liquid was then transferred into a sterile glass bijou with a pipette and the tip of the swab was broken into the bottle. Separate sterile blocks were used for each site. In the laboratory, the samples were vortexed and an aliquot of 0.5 ml was spread over Columbia blood agar plates (Oxoid, UK) with sterile pipettes and incubated at 37°C for at least 48 h. Some cultured samples were kept on plates at 37°C for 72 h if retrievals were performed on a Friday. This did not have an affect on the CFUs recovered.

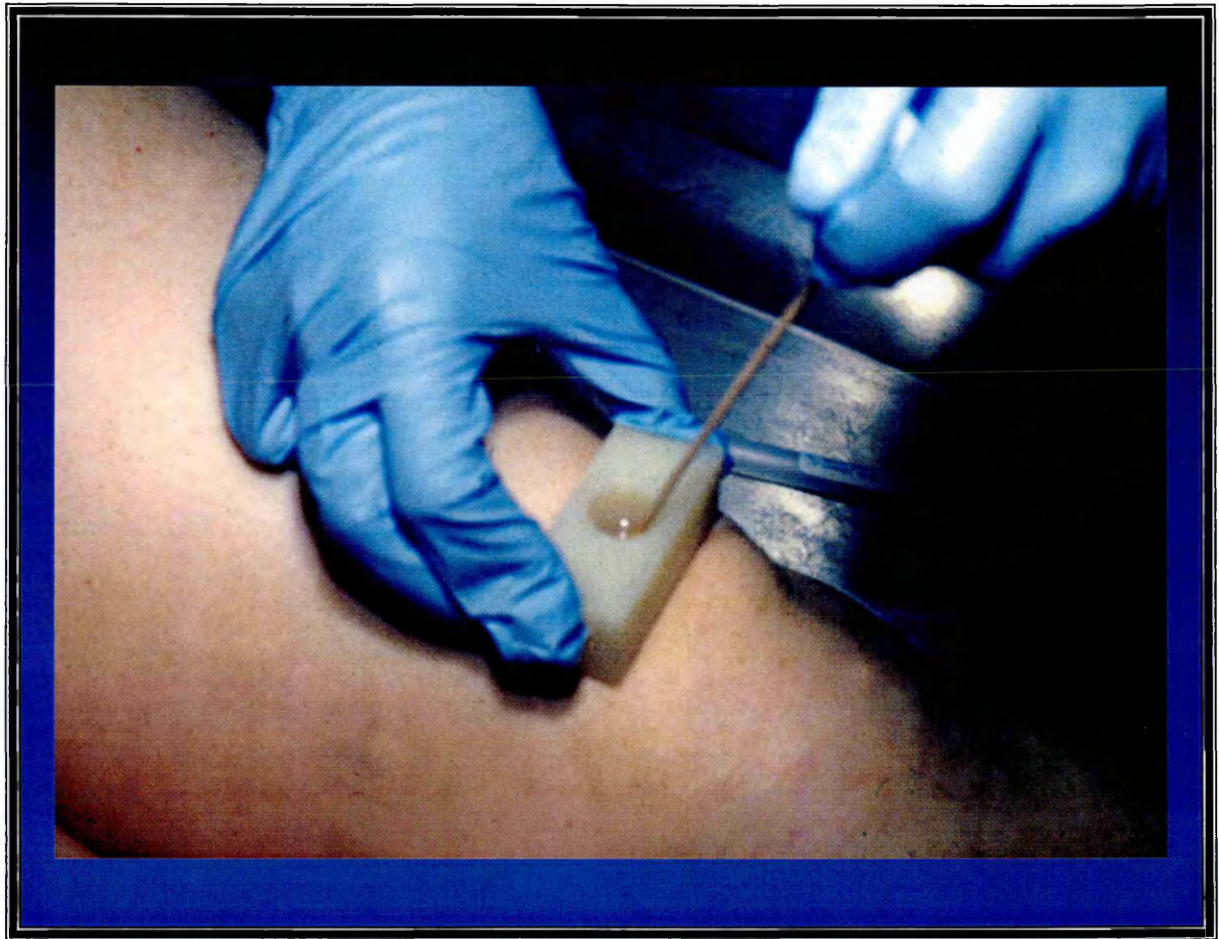


Figure 5. Cylindrical scrub technique applied on the donor's knee

2.2.3.2 Mortuary air:

During the retrieval period, 500 L of air were sampled from each side (10 cm away) of the donor and between donor and staff using a centrifugal air sampler containing Columbia base nutrient agar strips (Biotest, UK), (Fig. 6). Settle plates containing Columbia blood agar were also placed next to the instrument tray, donor's abdomen and foot area during the retrieval period (Figs. 7 & 8). The plates were incubated at 37°C for 48 h and for a further 48 h at room temperature.



Figure 6 An air sampler placed between the donor and retrieval staff.

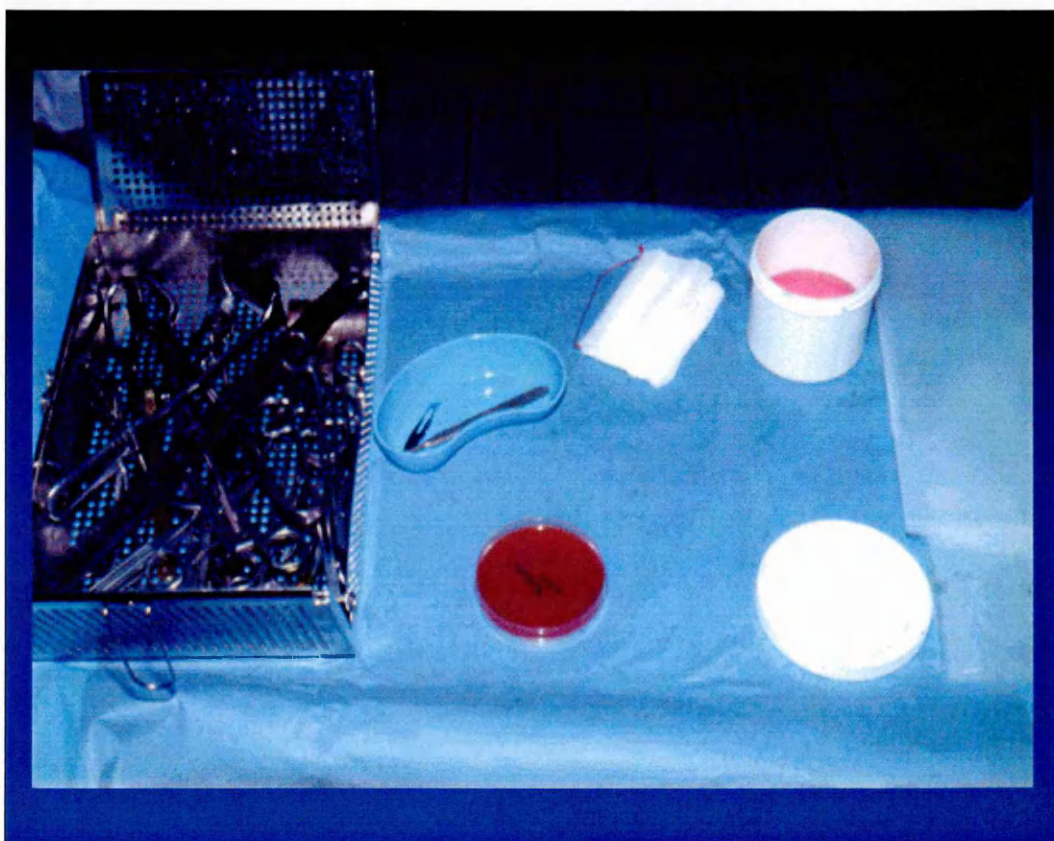


Figure 7 Settle plate placed on the sterile instrument field.



Figure 8. Settle plate placed on the donor's abdomen.

2.2.3.3 Retrieval staff

People shed very large numbers of skin particles ("squames"), a proportion of which will be contaminated by microcolonies of the bacteria and yeasts that grow on that individual's skin. Some of these will be trapped in clothing and some will be released into the air. Hoovering of the clothing will therefore reflect potential airborne contaminations. This sample gives an overall picture of the range of organisms dispersed from the whole body surface as the vast majority of bacteria recovered from clothing will have originated from the person's skin. There is likely to be less extraneous contamination in this sampling method than direct swabbing of a person's skin which may be transient and disproportionately represented. The body area that disperses most prolifically is the perineum. Indirect sampling, such as via clothing, is more socially acceptable than direct swabbing of intimate areas and thus more likely to gain a subject's consent (Noble WC. 1981, personal communication, Peter Hoffman).

The microbial flora of the retrieval staff was sampled on separate occasions (at retrieval staff meetings) by hoovering their own clothing with a Cassellar air slit sampler onto 5 inch Columbia blood agar plates for about 2 min. The plates were incubated for 48 h at 37°C in air and for a further 48 h at room temperature, to allow development of colonies. The colonial morphologies were recorded and about 10 different colonial appearances were observed. Colonies of different appearances were subcultured for purity on blood agar. Isolates were Gram stained, and tested for catalase and oxidase enzymes by standard methods. Gram positive cocci were identified to species level using the API ID32 STAPH (Biomériúx), and Gram negative bacteria were identified using the API 20E and 20NE microgalleries. Gram positive rods and other isolates were not identified to species level, but were grouped together as micrococci,

diphtheroids, *Propionibacterium* spp. and *Corynebacterium* spp. All isolates were stored on beads in glycerol at -70°C.

2.2.3.4 Retrieved tissue

Tissues were screened for bacterial contamination by swabbing the surface with a cotton swab (moistened with the PBS/Triton mixture) which was broken off into 3 ml of PBS/Triton. The swab was vortexed to release the bacteria and aliquots of 500 µL were cultured on Columbia blood agar at 37°C for at least 48 h and a further 48 h at room temperature. Colonies were subcultured for purity and subjected to species identification tests as above.

2.2.4 Stage 4-Matching bacterial isolates from sources and retrieved tissue

The colonial morphology of isolates from each retrieval (retrieval staff, tissue and donor's skin isolates) was compared together. The colony form was recorded from each plate and about 9 to 10 colonies of similar morphology were subcultured and identified to species level. Isolates of the same species from each retrieval were then subjected to further characterisation by molecular typing.

2.2.5 Stage 5-Confirmation of match between isolates

To confirm whether isolates recovered from the grafts, donor's skin, mortuary air and staff were genetically similar, sets of the same species from each retrieval were subjected to DNA fingerprinting by Random Amplification of Polymorphic DNA (RAPD).

2.2.6 Random amplification of polymorphic DNA (RAPD)

In order to identify the sources and routes of the transmission of bacteria to retrieved tissues some method of matching bacterial isolates has to be employed. It is not feasible to use typing methods specific for individual species as a wide range of organisms covering many species are likely to be encountered. The method chosen for this purpose must therefore be sufficiently comparative to allow matching of isolates of the same species. Phenotypic systems such as biotype are insufficiently discriminatory for this purpose and so a molecular based system is preferable. The obvious candidate among the numerous methods described for comparing bacterial genotypes are pulsed-field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD) analysis. The former method although offering the highest discrimination is unsuitable for routine use as it is labour intensive, has high equipment cost, and results may take up to a week to obtain. RAPD on the other hand has the advantage that is rapid and widely applicable and only a basic gel electrophoresis system is needed.

RAPD is based upon the *in vitro* amplification of random DNA fragments at low stringency using a single, short oligonucleotide primer of arbitrary sequence. The arbitrary primer sequence is short in length, typically 8-25 nucleotides, and will target unspecified genomic sequences which span from a priming site sequence to a nearby complementary sequence on each DNA strand. Amplification is enabled by very low stringency thermal cycling profiles, achieved by using annealing temperatures of about 33-50°C. This generates amplified products which are resolved electrophoretically, stained in ethidium bromide and visualised using a transilluminator. No prior knowledge of the template DNA is required to synthesise the oligonucleotide primers as is the case in conventional PCR amplification and the technique can be performed on virtually any organism. RAPD is therefore a simpler and more rapid procedure than other methods proposed for genetic analysis.

Ready-to-go[™] RAPD beads (Amersham Pharmacia Biotech, UK) were used to standardise the PCR process and optimise reproducibility. Two commercial primer sets were used (Amersham Pharmacia Biotech), primer 4-(5' d[AAGAGCCCGT]-3') for Gram-negative species and primer 6 (5' -d[CCC GTC AGC A] -3') for Gram-positive bacteria. Four to five colonies were suspended in 200 µl of sterile distilled water and boiled for 10 min to extract DNA. The PCR mixture was 2 µl of DNA extract, 18 µl of water and 5 µl of primer and 1 *Ready-to-go*[™] RAPD bead. Reactions were carried out in a thermocycler (Touchdown, Hybaid, UK) with the following stages: 95°C for 5 min-1 cycle, 95°C for 1 min, 36° for 1 min, 72°C for 2 min-45 cycles. PCR products were separated in 1.5 % Nusieve agarose (3:1 FMC Bioproducts, Flowgen, USA) at 100V for 3 h and stained with 1% ethidium bromide. The gel was viewed under ultra violet light and patterns were scanned and stored as TIFF. files. DNA profiles were compared with the aid of the Gel Compar software programme (Applied Maths, Belgium).

2.2.7 Statistical analysis:

Chi Square Test was carried out to determine significant associations between the rates of tissue contamination and donor and staff skin flora. A P value ≤ 0.05 indicated a significant difference.

2.3 Results

Twenty retrievals of cadaveric tissue were undertaken. Retrievals took place between March 1996 and December 1997. The causes of the donors' death are presented in Table 4. Two main causes of death were myocardial infarction and sub-arachnoid haematoma. There were also 7 unknown causes of death. The time between death and tissue retrieval varied from 7 h to 48 h in case 3. The average time between death and first incision was 16 h. Eight cadavers had had PMs of a duration between 30 min and 2.5 h (Table 4). The conditions of the mortuary and the activities where the retrieval took place are given in Table 5. The cleanliness of each mortuary was rated by a series of 1 to 3, with 3 being the least clean. Cleanliness was assessed by general tidiness, condition of the floors, age of the mortuary, how clean the mortuary tables were etc. Only six mortuaries were scored highest and the majority had some shortcomings with respect to cleanliness. Four centres were frankly unclean for the intended procedures. Half of all retrievals were performed as the sole activity in the mortuary at the time, in other retrievals, PM examination and embalment of cadavers were ongoing. On average, 3 to 4 staff (mortuary and retrieval) were present in the mortuary during the retrieval of tissue and in two centres, 7 or 8 individuals were present. For the majority of the donations (14), only the knees and the associated tissues were recovered; for the remainder tissues were retrieved from multiple sites. Table 6 shows that most retrievals were completed within 30 min approximately, but 3 took in excess of 1 h. In these cases skin was also retrieved, but this was not part of the planned study. Overall, there were 10 changes of air per hour in the mortuary, but one was claimed to be 30 changes of air per hour. Air changes were not measured due to time restriction and the information of air changes were provided by the mortuary technician and cannot be guaranteed to be accurate. Air samples were taken in 15 mortuaries with a range of 500-1000L.

Table 4. Information on donor and retrieval for each donation

Case	Cause of death	Date of retrieval	Time between death and first incision in h	PM prior to retrieval	Length of P.M in hours
1	Unknown	23/02/96	21	No	NR
2	RTA	24/04/96	20	No	NR
3	Hanging	26/04/96	48	Yes	1.15
4	Glyoma (died at surgery)	06/03/96	11.5	No	NR
5	Massive sub-dural haematoma	14/06/97	21	Yes	3.5
6	Arrhythmogenic RV dysplasia	15/07/97	21	Yes	1.5
7	Unknown	23/07/96	16	Yes	1.5
8	Myocardial infarction	10/10/96	21.5	Yes	0.5
9	Unknown	25/10/96	22	No	NR
10	Myocardial infarction	17/12/96	8	Yes	0.5
11	Unknown	15/07/98	10	No	NR
12	Unknown	03/09/97	12	No	NR
13	Myocardial infarction	15/05/97	10.5	No	NR
14	Sub-arachnoid haematoma	17/06/97	8.5	No	NR
15	Unknown	29/07/97	10	Yes	1.15
16	Sub-arachnoid haematoma	13/09/97	13	No	NR
17	Pulmonary embolism	01/10/97	14	Yes	1.5
18	Myocardial infarction	14/11/97	7	No	NR
19	Unknown	12/12/97	7.5	No	NR
20	Myocardial infarction	30/12/97	Unknown	No	NR

NR: Not relevant

Table 5. Information on mortuaries and retrieval for each donation

Cas e	Mortuary	Cleanliness of mortuary (1=good and 3=poor)	Activities before retrieval	Activities during retrieval	Number of staff before /during retrieval	Retrieval site
1	A	2	None	People entering in and out of the mortuary	5/4	Knees only
2	B	2	None	None	3/3	Full
3	C	1	2 PM	1 PM	2/4	Knees only
4	D	3	1 PM	People entering in and out of the mortuary	6/3	Knees only
5	E	2	2 PM, 1 embalment	Still performing PM, embalment	8/6	Knees only
6	F	1	1 PM	None	2/4	Full
7	G	2	2 full PM, 1 embalment	Embalment of 3 cadavers	7/5	Knees only
8	H	2	1 PM	None	2/5	Knees only
9	I	1	None	None	0/4	Full
10	H	2	None	None	0/3	Knees only
11	G	2	None	None	1/3	Knees only
12	J	3	None	None	0/3	Knees only
13	G	2	None	None	0/3	Full
14	K	3	1 PM	1 PM	5/ 4	Full
15	H	2	2 PM	None	3/3	Full
16	L	3	None	None	0/3	Knees only
17	F	1	2 PM, 1 of which was the donor	Mortuary staff observing PM	4/4	Knees only
18	M	2	3 PM	None	3/3	Knees only
19	N	1	None	None	2/3	Knees only
20	O	1	None	None	0/3	Knees only

NB: Full retrieval involves the removal of left (LKJ)and right knee joint (RKJ), left and right femoral shaft (LFS, RFS)and head (LFH, RFH), left and right achilles (LAT, RAT) and patellar tendons (LPT, RPT).

Knees only retrieval involves the removal of knee joints, femoral shaft and head only.

Table 6. Duration of retrieval and information on air microbiology of mortuaries for each donation

Case	Duration of retrieval (time between first incision and tissue put in bag)	Time air extractor switched on	Number of air changes/h	Volume of air sampled in litres/cubic metres	Number of CFU recovered from air before retrieval	Number of CFU recovered on the left side /right side of the donor during retrieval
1	30 min	Just prior to retrieval	10	500L	8	3/NT
2	1 h 50 min	Just prior to retrieval	10	NT	NT	NT/NT
3	10 min	Just prior to retrieval	10	NT	NT	NT/NT
4	33 min	Switches on when light switched on	10	500L	125	48/48
5	17 min	At all times	10	500L	20	8/8
6	37 min	Switched on at all times	10	NT	NT	NT/NT
7	30 min	Switched on at all times	10	500L	140	140/43
8	25 min	Not switched on	10	1000L	192	192/82
9	45 min	Not switched on	30?	1000L	121	254/183
10	31 min	Before arrival	10	500L	133	133/159
11	30 min	Not switched on	?	NT	NT	NT/NT
12	25 min	Not switched on	10	NT	NT	NT/NT
13	1 h, 23 min	Not switched on	10	1000L	86	113/184
14	50 min	Just prior to retrieval	?	1000L	33	38/127
15	1 h 40 min	½ an hour before retrieval	10	1000L	NT	NT/NT
16	25 min	Just prior to retrieval	10	500L	99	14/5
17	15 min	Switched on at all times	10	500L	46	9/11
18	15 min	Switched on arrival	20	500L	20	3/3
19	15 min	Just prior to retrieval	>10	500L	26	46/8
20	10 min	On arrival	10	500L	175	175/29

NT (not tested)

Table 7 shows that on average, 12 to 13 donations of knee joints were found to be contaminated on subsequent testing; the most commonly contaminated tissue was left femoral shafts, but this was not significant. Achilles and other tendons were retrieved from only 5 donors and were usually found to be contaminated.

A wide range of bacterial species were recovered from retrieved tissues and the donors' skin. Staphylococcal species, particularly coagulase-negative organisms were among the most prevalent (Table 8), but coryneforms, *Propionibacterium* and *Micrococcus* spp. were also common. Gram negative bacteria were infrequent with the exception of *E. coli*. Indeed, *E. coli* were recovered from 5 donors, and 3 of these had had a PM prior to the retrieval (Table 4). However, 5 of 8 subjects who had had a prior PM, did not yield coliforms on sampling. Only 2 of 20 retrievals yielded tissues with no bacterial growth on sampling. The number of different species identified in the tissues varied from 1 to 8 with the majority yielding at least 4 or more different species. The number of species on the donor skin varied from 1 to 9 and overall 2 to 3 matches in species identity was evident between donor tissue and skin. It is noteworthy that donor 16 yielded 9 species on the skin, 6 of which matched with the retrieved tissue.

Table 7. Type of tissue and bacterial contamination for individual donors

Case	RKJ	LKJ	RFS	LFS	LFH	RFH	RAT	LAT	RPT	LPT
1	-	-	-	-	-	-	NA	NA	NA	NA
2	+	+	+	+	+	+	-	-	-	-
3	+	+	+	-	+	-	NA	NA	NA	NA
4	-	-	-	-	-	-	NA	NA	NA	NA
5	+	-	NA	NA	NA	NA	NA	NA	NA	NA
6	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	NA	NA	NA	NA
8	+	+	+	+	+	+	NA	NA	NA	NA
9	+	-	-	+	-	+	+	-	-	-
10	+	+	+	+	-	+	NA	NA	NA	NA
11	+	+	+	+	+	+	NA	NA	NA	NA
12	+	-	+	+	-	+	NA	NA	NA	NA
13	-	+	-	+	+	-	+	+	+	+
14	NT	+	NT	+	+	NT	NT	NT	NT	NT
15	+	+	+	+	-	+	+	+	+	+
16	-	+	-	+	+	+	NA	NA	NA	NA
17	+	+	+	+	+	+	NA	NA	NA	NA
18	-	+	+	+	+	+	NA	NA	NA	NA
19	+	-	-	-	-	-	NA	NA	NA	NA
20	-	-	+	+	+	+	NA	NA	NA	NA
	13/19	13/2	12/18	15/19	12/19	13/18	4/5	2/5	3/5	3/5

NB: + (Bacterial contamination), -(negative swab culture), NT (not tested), NA (tissues that are not retrieved). RKJ & LKJ: Right & left Knee Joints; RFS & LFS: Right & Left Femoral Shafts; LFH & RFH: Left & Right femoral heads; RAT & LAT: Right and Left Achilles Tendons; RPT & LPT: Right & Left Patellar Tendons.

Table 8 List of species isolated from tissue and donor of each retrieval

case	Species isolated from tissue	Species isolated from donor's skin	No of matching species between tissue and donor
1	No growth	<i>S. epidermidis</i> <i>S. warneri</i> <i>Corynebacterium</i> spp.	0
2	<i>E. coli</i> <i>S. cohnii</i> <i>S. epidermidis</i> <i>S. hominis</i>	<i>E. coli</i> <i>S. capitis</i> <i>S. epidermidis</i> <i>S. hominis</i> <i>S. simulans</i> <i>S. warneri</i>	3
3	<i>Bacillus</i> spp. <i>Corynebacterium</i> spp. <i>S. capitis</i> <i>S. epidermidis</i>	<i>Acinetobacter</i> spp. <i>Corynebacterium</i> spp. <i>S. capitis</i> <i>S. epidermidis</i> <i>S. haemolyticus</i>	3
4	No growth	<i>S. epidermidis</i> , <i>S. capitis</i> <i>Acinetobacter</i> spp. <i>Propionibacterium</i> spp.	0
5	<i>S. epidermidis</i>	<i>S. epidermidis</i>	0
6	<i>E. coli</i> <i>S. capitis</i> <i>S. epidermidis</i> <i>S. warneri</i>	<i>E. coli</i> <i>Propionibacterium</i> spp. <i>S. capitis</i> , <i>S. epidermidis</i> <i>S. hominis</i>	3
7	<i>S. capitis</i> <i>S. epidermidis</i> <i>S. haemolyticus</i> <i>S. hominis</i>	<i>S. capitis</i> <i>S. epidermidis</i>	2

Table 8 **List of species isolated from tissue and donor of each retrieval**

case	Species isolated from tissue	Species isolated from donor's skin	No of matching species between tissue and donor
1	No growth	<i>S. epidermidis</i> <i>S. warneri</i> <i>Corynebacterium</i> spp.	0
2	<i>E. coli</i> <i>S. cohnii</i> <i>S. epidermidis</i> <i>S. hominis</i>	<i>E. coli</i> <i>S. capitis</i> <i>S. epidermidis</i> <i>S. hominis</i> <i>S. simulans</i> <i>S. warneri</i>	3
3	<i>Bacillus</i> spp. <i>Corynebacterium</i> spp. <i>S. capitis</i> <i>S. epidermidis</i>	<i>Acinetobacter</i> spp. <i>Corynebacterium</i> spp. <i>S. capitis</i> <i>S. epidermidis</i> <i>S. haemolyticus</i>	3
4	No growth	<i>S. epidermidis</i> , <i>S. capitis</i> <i>Acinetobacter</i> spp. <i>Propionibacterium</i> spp.	0
5	<i>S. epidermidis</i>	<i>S. epidermidis</i>	0
6	<i>E. coli</i> <i>S. capitis</i> <i>S. epidermidis</i> <i>S. warneri</i>	<i>E. coli</i> <i>Propionibacterium</i> spp. <i>S. capitis</i> , <i>S. epidermidis</i> <i>S. hominis</i>	3
7	<i>S. capitis</i> <i>S. epidermidis</i> <i>S. haemolyticus</i> <i>S. hominis</i>	<i>S. capitis</i> <i>S. epidermidis</i>	2

Table 8 continued. List of species isolated from tissue and donor of each retrieval

case	Species isolated from tissue	Species isolated from donor's skin	No of matching species between tissue and donor
8	<i>Aerococcus viridans</i> <i>E. coli</i> <i>Micrococcus</i> spp. <i>S. capitis</i> <i>S. epidermidis</i> <i>S. salivarius</i>	<i>S. capitis</i> <i>E. coli</i>	2
9	<i>S. capitis</i> <i>S. epidermidis</i> <i>S. hominis</i> <i>S. warneri</i>	<i>S. haemolyticus</i>	0
10	GNR <i>Micrococcus</i> spp. <i>S. aureus</i> <i>S. epidermidis</i> <i>S. haemolyticus</i>	<i>Micrococcus</i> spp. <i>S. aureus</i> <i>S. epidermidis</i>	2
11	<i>Acinetobacter</i> spp. <i>Branhamella</i> spp. <i>Corynebacterium</i> spp. <i>Micrococcus</i> spp. <i>Propionibacterium</i> spp. <i>S. capitis</i> <i>S. epidermidis</i> <i>S. lugdenensis</i> <i>S. saprophyticus</i>	<i>Micrococcus</i> spp. <i>Propionibacterium</i> spp. <i>S. epidermidis</i>	3
12	<i>A. viridans</i> <i>Bacillus</i> spp. <i>Micrococcus</i> spp. <i>S. lentus</i>	<i>S. lentus</i>	1

Table 8 continued. List of species isolated from tissue and donor of each retrieval

case	Species isolated from tissue	Species isolated from donor's skin	No of matching species between tissue and donor
13	<i>E. coli</i> <i>Micrococcus</i> spp. <i>Propionibacterium</i> spp. <i>S. cohnii</i> <i>S. epidermidis</i> <i>S. hominis</i>	<i>S. capitis</i> <i>S. haemolyticus</i> <i>S. hominis</i> <i>S. lugdenensis</i>	1
14	<i>Corynebacterium</i> spp. <i>Micrococcus</i> spp. <i>S. capitis</i> <i>S. epidermidis</i>	<i>Micrococcus</i> spp. <i>S. capitis</i> <i>S. saprophyticus</i>	2
15	<i>Micrococcus</i> spp. <i>S. capitis</i> <i>S. epidermidis</i> <i>S. hominis</i> <i>S. saprophyticus</i> <i>S. warneri</i> <i>Staphylococcus</i> spp.	<i>S. capitis</i> <i>S. epidermidis</i> <i>S. hominis</i> <i>S. warneri</i>	4
16	<i>Corynebacterium</i> spp. <i>S. capitis</i> <i>S. epidermidis</i> <i>S. hominis</i> <i>S. haemolyticus</i> <i>Micrococcus</i> spp.	<i>Bacillus</i> spp. <i>Corynebacterium</i> spp. <i>Micrococcus</i> spp. <i>S. capitis</i> <i>S. epidermidis</i> <i>S. hominis</i> <i>S. lugdenensis</i> <i>S. warneri</i> <i>S. haemolyticus</i>	6

Table 8 continued. List of species isolated from tissue and donor of each retrieval

case	Species isolated from tissue	Species isolated from donor's skin	No of matching species between tissue and donor
17	<i>Acinetobacter</i> spp. <i>Corynebacterium</i> spp. <i>E. coli</i> <i>S. chromogenes</i>	<i>Corynebacterium</i> spp. <i>Diphtheroids</i> <i>E. coli</i> <i>S. epidermidis</i> <i>S. simulans</i> <i>S. warneri</i>	2
18	<i>Bacillus</i> spp. <i>Micrococcus</i> spp. <i>S. haemolyticus</i>	<i>Micrococcus</i> spp. <i>S. capitis</i> <i>S. epidermidis</i> <i>S. haemolyticus</i>	2
19	<i>S. epidermidis</i>	<i>S. epidermidis</i>	1
20	<i>Micrococcus</i> spp. <i>S. haemolyticus</i>	<i>Acinetobacter</i> spp. <i>S. haemolyticus</i>	1

Table 9. Species isolated according to type of tissue

Type of tissue	Femoral head	Femoral shaft	Knee joint	Patellar Tendon	Achilles tendon
Species isolated	<i>Acinetobacter</i> spp.	<i>Acinetobacter</i> spp.	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>
	<i>Aerococcus viridans</i>	<i>Aerococcus viridans</i>	GNR	<i>Propionibacterium</i> spp.	<i>S. capitis</i>
	<i>Branhamella</i> spp.	<i>Bacillus</i> spp.	<i>Micrococcus</i> spp.	<i>S. cohnii</i>	<i>S. epidermidis</i>
	<i>Corynebacterium</i> spp.	<i>Branhamella</i> spp.	<i>Propionibacterium</i> spp.	<i>S. epidermidis</i>	<i>S. warneri</i>
	<i>E. coli</i>	<i>Corynebacterium</i> spp.	<i>S. aureus</i>		
	<i>M. luteus</i>	<i>E. coli</i>	<i>S. capitis</i>		
	<i>Micrococcus</i> spp.	<i>Micrococcus</i> spp.	<i>S. chomogenes</i>		
	<i>Propionibacterium</i> spp.	<i>Propionibacterium</i> spp.	<i>S. cohnii</i>		
	<i>S. capitis</i>	<i>S. capitis</i>	<i>S. epidermidis</i>		
	<i>S. epidermidis</i>	<i>S. epidermidis</i>	<i>S. haemolyticus</i>		
	<i>S. haemolyticus</i>	<i>S. haemolyticus</i>	<i>S. saprophyticus</i>		
	<i>S. hominis</i>	<i>S. hominis</i>			
	<i>S. lentus</i>	<i>S. lentus</i>			
	<i>S. salivaris</i>	<i>S. lugdenensis</i>			
	<i>S. saprophyticus</i>				

Table 10. Organisms recovered from clothing of staff

Staff	A	B	C	D	E	F
Species isolated	<i>Micrococcus</i> spp.	<i>Bacillus</i> spp.	<i>Bacillus</i> spp.	<i>Acinetobacter</i> spp.	<i>Acinetobacter</i> spp.	<i>Acinetobacter</i> spp.
	<i>S. aureus</i>	<i>Micrococcus</i> spp.	<i>Corynebacterium</i> spp.	<i>Corynebacterium</i> spp.	<i>Bacteroides</i> spp.	<i>Micrococcus</i> spp.
	<i>S. cohnii</i>	<i>S. capitis</i>	<i>Micrococcus</i> spp.	<i>Micrococcus</i> spp.	<i>Micrococcus</i> spp.	<i>S. capitis</i>
	<i>S. epidermidis</i>	<i>S. cohnii</i>	<i>S. cohnii</i>	<i>S. haemolyticus</i>	<i>S. epidermidis</i>	<i>S. cohnii</i>
	<i>S. haemolyticus</i>	<i>S. epidermidis</i>	<i>S. epidermidis</i>		<i>S. hominis</i>	<i>S. haemolyticus</i>
	<i>S. hominis</i>	<i>S. haemolyticus</i>	<i>S. haemolyticus</i>			
	<i>S. kloosii</i>	<i>S. hominis</i>	<i>S. hominis</i>			
	<i>S. warneri</i>	<i>S. kloosii</i>	<i>S. kloosii</i>			
		<i>S. saprophyticus</i>	<i>Staphylococcus</i> spp.			
		<i>S. warneri</i>				

Table 10. continued, Organisms recovered from clothing of staff

Staff	G	H	I	J	K	L
Species isolated	<i>Acinetobacter</i> spp.	<i>Acinetobacter</i> spp.	<i>Micrococcus</i> spp.	<i>Acinetobacter</i> spp.	<i>Acinetobacter</i> spp.	<i>Acinetobacter</i> spp.
	<i>Aeromonas hydrophila</i>	<i>Bacillus</i> spp.	<i>S. capitis</i>	<i>Aeromonas hydrophila</i>	<i>Bacillus</i> spp.	<i>Bacillus</i> spp.
	<i>Bacillus</i> spp.	<i>Micrococcus</i> spp.	<i>S. cohnii</i> ,	<i>Bacillus</i> spp.	<i>Micrococcus</i> spp.	<i>M. luteus</i>
	<i>Micrococcus</i> spp.	<i>S. capitis</i>	<i>S. epidermidis</i>	<i>Corynebacterium</i> spp.	<i>S. cohnii</i>	<i>Micrococcus</i> spp.
	<i>Pseudomonas stutzeri</i>	<i>S. cohnii</i>	<i>S. haemolyticus</i>	<i>Micrococcus</i> spp.	<i>S. epidermidis</i>	<i>S. capitis</i>
	<i>S. haemolyticus</i>	<i>S. haemolyticus</i>	<i>S. hominis</i>	<i>S. capitis</i>	<i>S. haemolyticus</i>	<i>S. cohnii</i>
	<i>S. hominis</i>	<i>S. hominis</i>		<i>S. hominis</i>	<i>S. warneri</i>	<i>S. haemolyticus</i>
	<i>S. kloosii</i>	<i>Staphylococcus</i> spp.		<i>S. warneri</i>	<i>Staphylococcus</i> spp.	<i>Staphylococcus</i> spp.

M, N, O and P are other member of staff that was not sampled for their skin microflora due to their unavailability

Isolates of the same species from single retrievals were compared by RAPD PCR to determine the degree of matching of DNA fingerprints. The similarity of isolates was displayed as a dendrogram and a cut-off point of 85% was taken to distinguish different strain populations. Therefore, isolates which showed greater than 85% similarity in DNA pattern were considered to be genetically related. Table 11 shows the degree of matching of isolates from tissues with donor skin, isolates retrieved from staff and from the air samples. In addition, on 6 occasions, staff isolates were compared with those from the air samples. Concerning tissue and donor isolates, 15 pairs of isolates were classified as related and all but 3 pairs were staphylococci or micrococci; the other pairs were all identified as *E. coli* and these had similarity values above 90%. *S. aureus* isolated from donor's skin and tissue from a particular retrieval also had similarity values above 90%. This also applied to *S. capitis* in another incidence. For tissue and staff isolates comparison, 10 matches had 85% or greater similarity, but 7 others fell between 80-85% and could not be unequivocally classified as different strains. There were notably fewer matches (5) found between isolates of the same species from the retrieved tissue and air contaminants. Matches were also found for 4 of the 6 pairs of staff and air isolates. Examples of RAPD profiles, along with dendrograms of matching isolates are shown in Figs. 9-14. Figs. 9 and 10 show an example where 9 isolates of *S. epidermidis* cluster above 90% similarity, with the remainder of the isolates having as little as 30% similarity. These were therefore considered to represent distinct strains. Figs. 11 and 12 show tight clustering of above 90% or greater for 7 isolates of *E. coli* recovered from retrieved tissue and settle plates that were placed at the foot end of the donor. Figs. 13 & 14 also show a tight clustering of above 90% for 4 *E. coli* isolates. These isolates were recovered from tissue and donor's skin before skin disinfection.

The number of DNA matches sorted by individual species are summarised in Table 12. It shows that *S. epidermidis* was the most homogeneous of the species commonly isolated. Other coagulase-negative species (*S. capitis*, *S. warneri*, *S. hominis* and *S. haemolyticus*) gave relatively low matches and were comprised mainly of genetically distinct strains. Heterogeneous populations were also revealed in the micrococci by DNA fingerprinting.

There was no significant difference between donor and staff as the common source of contamination ($P \geq 0.05$), and so both staff and donor probably contributed equally to the contamination of tissue. There were only 5 cases of identical matches of organisms isolated from air and tissue, whereas donor vs tissue and staff vs tissue had 16 and 11 matches between organisms respectively. There was a significant differences between air vs staff and donor ($P \leq 0.05$).

Table 11: Number of cases with matching species and identical DNA fingerprints

NT: Not tested

NA: no matching of species within retrieval

NM: No match, percentage similarity below 85%

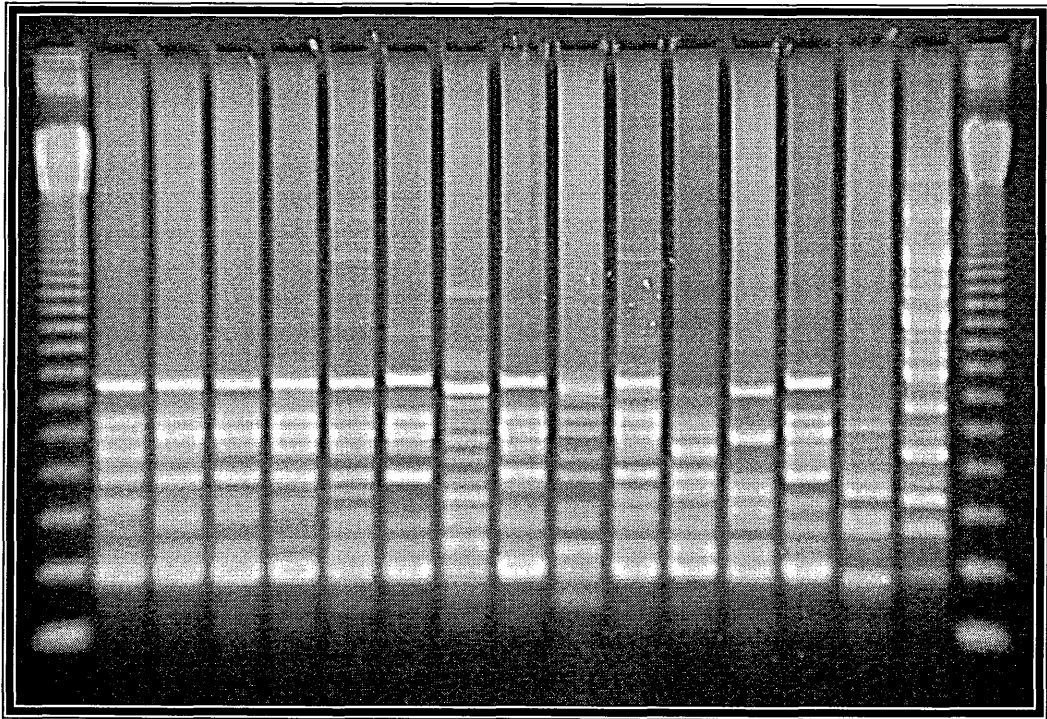
Case	Species	Tissue vs. donor isolates	Tissue vs. staff isolates	Tissue vs. air isolates	Staff vs. air isolates
2	<i>S. epidermidis</i>	NM	NM	NA	NT
	<i>E. coli</i>	NT	NT	NT	NT
	<i>S. hominis</i>	NM	NM	NM	NM
3	<i>S. capitis</i>	97%	NM	NM	NT
	<i>S. epidermidis</i>	NM	NM	NM	NM
	<i>Corynebacterium</i> spp.	NT	NT	NA	NA
	<i>S. hominis</i>	NA	NA	NA	NM
5	<i>S. epidermidis</i>	100%	NM	97%	NM
	<i>S. haemolyticus</i>	NA	NA	NA	NM
6	<i>E. coli</i>	100%	NA	NA	NA
	<i>S. epidermidis</i>	NM	NM	NA	NA
	<i>S. capitis</i>	100%	NM	NA	NA
	<i>S. hominis</i>	NA	NA	NA	NA
	<i>S. warneri</i>	NA	NA	NM	NA
7	<i>S. epidermidis</i>	97%	86%	NA	NM
	<i>S. capitis</i>	NM	91%	NA	NA
	<i>S. haemolyticus</i>	NA	NA	NM	NM
	<i>S. warneri</i>	NA	NA	NA	NM
8	<i>E. coli</i>	100%	NA	91%	NA
	<i>S. epidermidis</i>	NA	97%	NM	82%
	<i>S. capitis</i>	NM	NM	NM	NM
9	<i>S. epidermidis</i>	NA	86%	NM	NA
	<i>S. warneri</i>	NA	NM	NM	NM
	<i>S. hominis</i>	NA	NA	NM	NA
	<i>S. capitis</i>	NA	NA	NM	95%

Table 11 continued: Number of cases with matching species and identical DNA fingerprints

Case	Species	Tissue vs. donor isolates	Tissue vs. staff isolates	Tissue vs. air isolates	Staff vs. air isolates
10	<i>S. aureus</i>	100%	NA	NA	NA
	<i>S. epidermidis</i>	89%	81%	87%	NA
	<i>S. haemolyticus</i>	NA	85%	NA	NA
	<i>Micrococcus</i> spp.	85%	NM	NM	NM
11	<i>S. epidermidis</i>	NT	86%	NA	NM
	<i>S. saprophyticus</i>	NA	NM	NA	NA
	<i>Micrococcus</i> spp.	85%	NM	NA	NA
	<i>S. capitis</i>	NA	86%	NA	NA
	<i>Propionibacterium</i> spp.	85%	NA	NA	NA
12	<i>S. lentus</i>	90%	NA	NA	NA
	<i>S. hominis</i>	NA	NA	NA	97%
	<i>S. epidermidis</i>	NA	NA	NA	NM
13	<i>S. hominis</i>	NM	NM	NM	NM
	<i>S. epidermidis</i>	NA	91%	NM	NM
	<i>S. haemolyticus</i>	NA	NA	NA	91%
14	<i>Micrococcus</i> spp.	NM	91%	94%	90%
	<i>S. epidermidis</i>	NA	NM	NA	NA
	<i>S. hominis</i>	NA	NA	NA	NM
	<i>S. capitis</i>	NT	NA	NA	NA
15	<i>S. epidermidis</i>	94%	NM	NM	NA
	<i>S. capitis</i>	NM	NM	NM	NA
	<i>S. warneri</i>	NM	NM	NM	NA
	<i>S. hominis</i>	NM	NM	NA	NA
	<i>S. saprophyticus</i>	NA	NM	NA	NA

Table 11 continued: Number of cases with matching species and identical DNA fingerprints

Case	Species	Tissue vs. donor isolates	Tissue vs. staff isolates	Tissue vs. air isolates	Staff vs. air isolates
16	<i>S. haemolyticus</i>	NM	NA	NM	NA
	<i>S. epidermidis</i>	NM	89%	NA	NA
	<i>Corynebacterium</i> spp.	NM	NA	NA	NA
	<i>S. capitis</i>	NM	NM	NT	NT
	<i>Micrococcus</i> spp.	NT	NT	NT	NT
	<i>S. hominis</i>	NM	NM	NT	NA
17	<i>E. coli</i>	100%	NA	NA	NT
	<i>Corynebacterium</i> spp.	NM	NT	NT	NT
18	<i>Micrococcus</i> spp.	NM	NM	NM	NA
	<i>S. haemolyticus</i>	NM	NM	NM	NA
	<i>S. epidermidis</i>	NA	NA	NA	NM
19	<i>S. epidermidis</i>	95%	94%	95%	94%
20	<i>S. haemolyticus</i>	87%	NA	NA	NA
	<i>Micrococcus</i> spp.	NA	NM	NA	NA
		16/35 46%	11/36 31%	5/25 20%	4/23 17%



Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Figure 9. RAPD gel of *S.epidermidis* from case 093010w5; lane 1: ladder (123bp markers); lane 2: (RKJ) right knee joint; lane 3: right knee swab (RKS); lane 4, RKS 2 ; lane 5: RKS 3; lane 6: RKS 4; lane 7: AS Bret 1 (air sampling before retrieval); lane 8: AS Bret 5; lane 9: AS-SF 1 (Air sampling on near sterile field); lane10: AS-SF 3; lane 11: AS-SF 4; lane 12: SP-SF 1 (settle plate on sterile field); lane 13: SP-SF 2; lane 14: ASB 1 (air sampling bottom end of donor during retrieval); lane 15: blank (water control); lane 16:positive control (*E. coli*); lane 17: ladder (123bp markers).

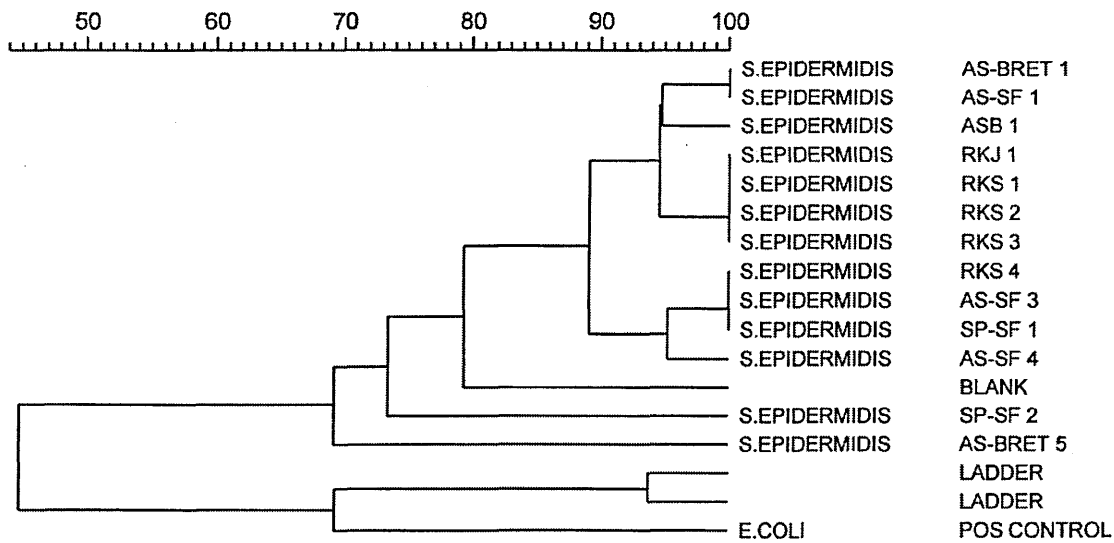
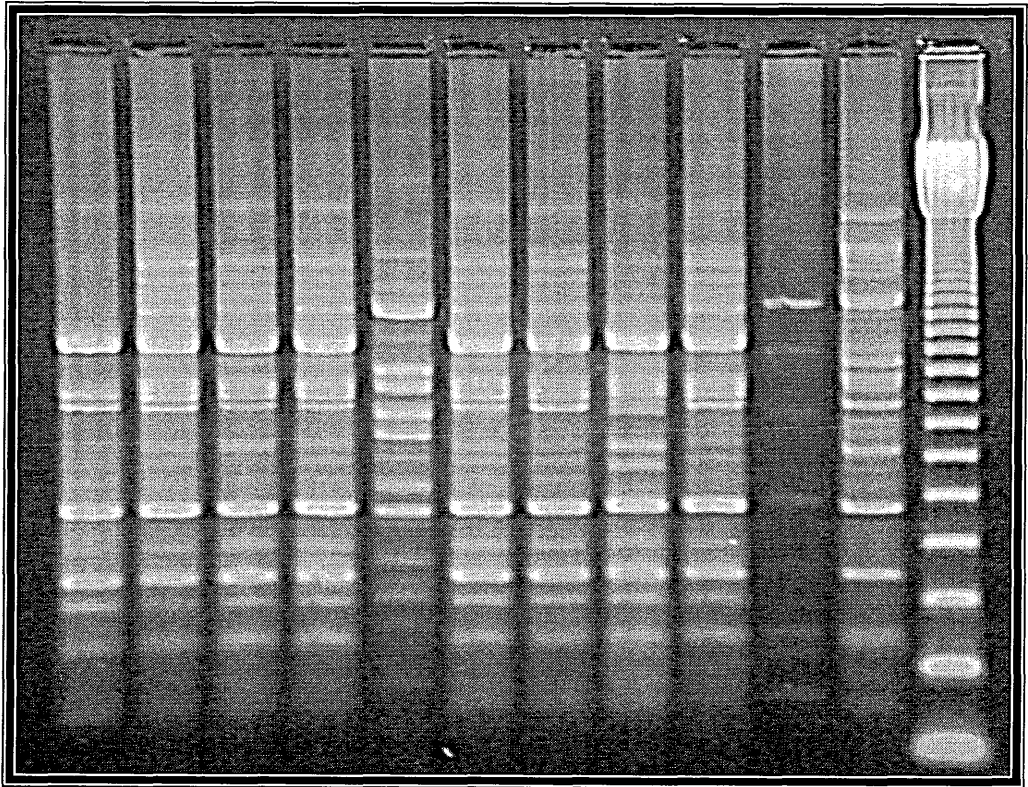


Figure 10. Dendrogram showing the clustering of *S. epidermidis*



Lane 1 2 3 4 5 6 7 8 9 10 11 12

Figure 11. RAPD gel of *E. coli* from case 093170w5; lane 1: RFH (right femoral head); lane 2: LKJ 1 (left knee joint); lane 3: LFS (left femoral shaft); lane 4: LFH 1 (left femoral head); lane 5: LFH 2; lane 6: RKJ 1 (right knee joint); lane 7: RKJ 2; lane 8: LKJ 3; lane 9: SP-BOT (settle plate positioned at the foot end of the donor); lane 10: blank (water control); lane 11: positive control (*E. coli*); lane 12: ladder (123bp marker)

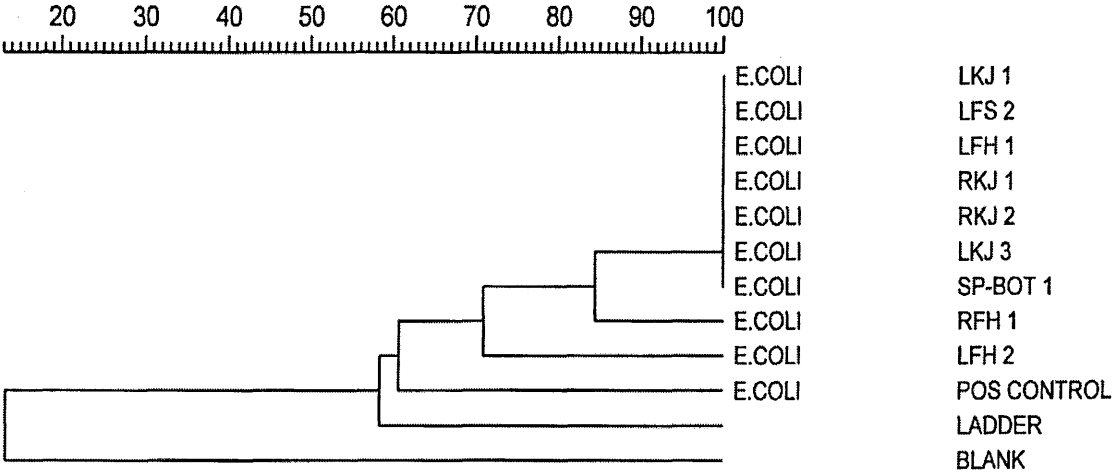


Figure 12. Dendrogram showing the clustering of *E. coli* from case 093010w5

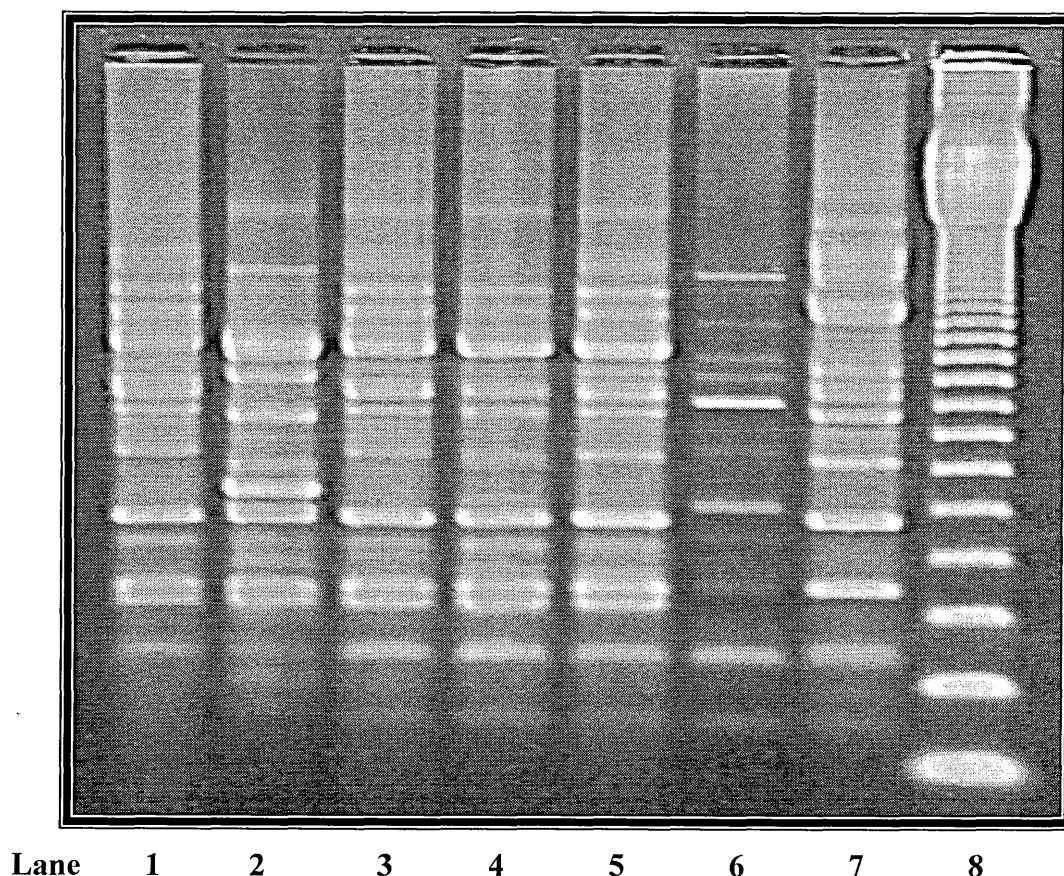


Figure 13. RAPD of *E. coli* from case 093560W3; lane 1: LAT 1 (left achilles tendon); lane 2: LFS 1 (left femoral shaft); lane 3: LKJ 1 (left knee joint); lane 4: LKS (donors left knee swab); lane 5: RTS (donors right thigh skin swab); lane 6: blank (water control); lane 7: control (positive control, *E. coli*); lane 8: ladder (123 bp marker).

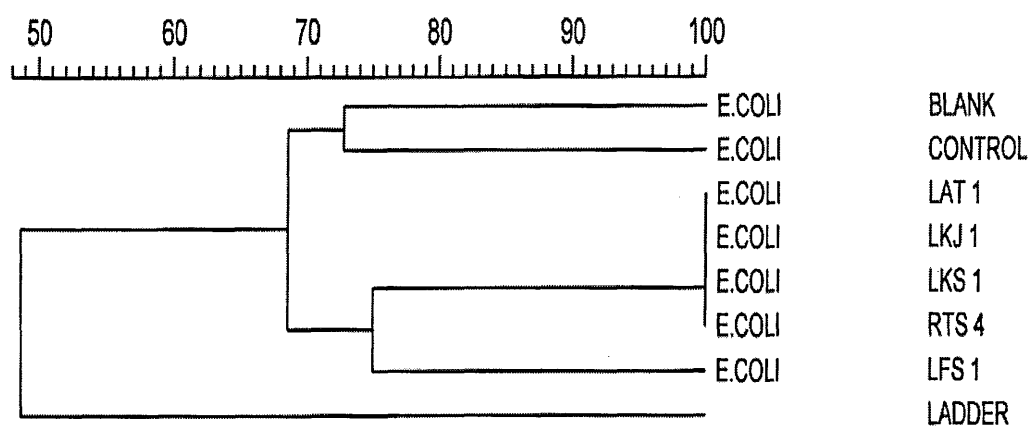


Figure 14. Dendrogram showing the clustering of *E. coli* from case 093560W3

Table 12. Summary of the number of cases with matching species and identical DNA fingerprints:

Organisms isolated	Total
<i>S. epidermidis</i>	16/40 (40 %)
<i>S. capitis</i>	4/19 (23 %)
<i>S. warneri</i>	0/10 (0 %)
<i>S. hominis</i>	1/16 (6 %.)
<i>S. haemolyticus</i>	2/11 (18 %)
<i>S. aureus</i>	1/1 (100 %)
<i>S. lentus</i>	1/1 (100 %)
<i>S. saprophyticus</i>	0/1 (0 %)
<i>E. coli</i>	4/4 (100 %)
<i>Micrococcus</i> spp.	5/14 (36 %)
<i>Propionibacterium</i> spp.	1/1 (100 %)
<i>Corynebacterium</i> spp.	0/2 (0 %)
Total	35/120

2.4 Discussion and conclusion

2.4.1 Influence of the environment on bacterial contamination of cadaveric tissue

Most cadaveric retrievals are performed within a mortuary where the layout and the environmental conditions are significantly different to those of an operating room (OR). An OR is normally enclosed from the outside with environmental conditions strictly controlled. The numbers of bacteria within the room that have HEPA filtered systems are controlled to levels of $<10 \text{ CFU/m}^3$ to protect surgical wounds and instruments from contamination (Gosden *et al.* 1998, Hambræus 1988, Babb *et al.* 1995, Fox & Whyte 1995, Humphreys *et al.* 1995).

Within the OR, effective theatre disciplines are also applied to minimise contamination. These include restriction on the number of staff present, human traffic in and out of the room, disinfection of equipment and the surrounding area prior to surgery, and doors remaining closed during surgery (Dennis *et al.* 1976). In contrast, the environmental conditions in mortuaries are not controlled to prevent bacterial contamination of cadaveric tissue. The room is usually open for traffic of mortuary technicians and other members of staff. There are also no restrictions on the number of staff present and the ventilation system may vary considerably from non-existent to excellent. Where a ventilation system exists, it may consist of a simple extraction fan with no supply of clean air, or a 'Cross-Flow' extraction system with fewer than 10 air changes per hour. Indeed, a study on the microbiology of mortuaries by Newsom *et al.* (1983) revealed that in one particular mortuary a ventilation system did not exist and during busy periods it was occupied by up to 7 staff.

The ventilation system of mortuaries visited in this study varied, with an average air change per hour of 10, however, in several cases, the fans were not switched on (Table 6) during retrieval, which should contribute to a higher air bacterial count (Newsom *et al.* 1983). However, an increase in air bacterial count when staff members entered the

room while the extraction system was switched off as in case 8 could not be demonstrated as the air count remained the same before and during the retrieval. Ventilation systems within mortuaries are designed primarily to protect the staff from contracting potential diseases such as tuberculosis that may be disseminated in the general surroundings during a post-mortem (Grist and Emslie 1978, 1985, 1989). Multiple activities can also take place at the same time as tissue retrieval. Staff may enter and leave the room, thus contributing to higher levels of bacteria in the air. Indeed, it has been shown that the air counts can increase from 25 to 100 cfu/350L as a result of an individual entering the room (Newsom *et al.* 1983).

There is no air pressure difference between the outside and the inside of mortuaries. Ideally, as for operating rooms, a positive pressure should exist inside the room to prevent organisms entering from the outside. Surprisingly, the opposite was suggested over 20 years ago by Newsom *et al.* (1983) who considered that there should be a slight negative pressure inside the mortuary in order to contain odours. The general surroundings in mortuaries are usually contaminated with bacteria and for example, necropsy tables are commonly heavily contaminated with Gram negative bacilli since they may only be cleaned and not disinfected (Babb *et al.* 1989).

A full PM is regularly performed in the mortuary prior to or during a retrieval and it is known that potentially pathogenic organisms such as *E. coli* and *Clostridium perfringens* are released into the air as aerosols which eventually settle on surfaces (Newsom *et al.* 1983; Babb *et al.* 1989; Al-wali *et al.* 1993, Newsom *et al.* 1983) found coliforms to be the highest in numbers in mortuaries that have none or only poor ventilation.

The combination of multiple activities and unrestricted levels of staff can lead to a high level of bacteria in the air which will potentially be disseminated among the staff attending a PM. The rate of tissue contamination should therefore be higher than surgical wound contamination in OR. Conditions in mortuaries seem to be directly reflected by the rate of tissue contamination, as much as 68 % of retrieved tissues in this study were found to be contaminated despite the use of aseptic techniques such as staff wearing surgeon's gowns, caps etc. Contamination could possibly arise from within the gut flora and also be disseminated from the large blood vessels into the peripheral tissues. Of the 68% of tissues found to be contaminated in this study up to 50% had isolates that matched with either donor or staff. The remaining were not of the same species or did not match by RAPD. There is a possibility that these isolates could have come from elsewhere in the mortuary. Bettin *et al.* (1998) carried out a study on the incidence of pathogenic microorganisms in bone allografts retrieved in the mortuary, and found that the rate of contamination was in fact lower than that for tissue retrieved within the operating room. However, most importantly, a higher rate of tissues with highly pathogenic organisms was recovered from donors in the mortuary compared to operating room donors (60% and 33% respectively). In both cases, identical retrieval procedures and bacteriological methods were used. Types of organisms that were considered highly pathogenic in Bettin's study were *S. aureus*, *Enterococcus*, *E. coli*, *Candida*, *Bacteriodes*, spores, *Acinetobacter*, *Alcaligenes*, and *Pseudomonas* spp.

2.4.2 Contamination of tissue by staff and donor's skin flora

Many investigations have identified the routes and means of contamination of surgical wounds in the OR setting. These include i) the air, through direct settling of bacteria or indirectly via instruments and gloves (Hambræus 1988, Whyte *et al.* 1992, Fox & Whyte 1995), ii) by direct contact with clothes or from punctured gloves, (Whyte *et al.*

1982, 1991) and iii) from the patient's skin (Benedikstordottir and Kolstad 1984). Nevertheless, despite the differences in environmental conditions and practices in OR and PM rooms, the sources of contamination remain the same, but result in markedly different rates of contamination. This study has shown that both staff and donor are able to contaminate tissue directly and indirectly through air. *S. epidermidis* recovered from staff and air (Table 11, case 5) and *S. epidermidis* recovered from tissue and staff (Table 11, case 8) were found to be identical strains in each case (97% and 94% respectively).

The evidence of indirect contamination by staff is seen in two particular cases (Table 11). In case 14, an identical strain of *Micrococcus* spp. was recovered from tissue, staff and settle plates placed on the instrument field, and in case 19, an identical strain of *S. epidermidis* was only recovered from settle plates and staff. These results indicate that instruments became contaminated during retrieval and it is difficult to see how this can be prevented. In practice, for this study a team of 3 or 4 persons would simultaneously or consecutively set out instruments, disinfecting the skin of the donor and drape the appropriate areas before the retrieval. It was not our practice to cover instruments during the procedure. The instrument field is set up prior to donor preparation and remains exposed to the mortuary air during the entire retrieval. Brown *et al.* (1996) found that the bacterial air count within the OR increased four-fold during skin preparation and draping of patients undergoing joint replacement, despite the use of ultra-clean ventilation. These findings were also supported by Deijkers *et al.* (1997), who found that during donor and back table preparation, the air count increased substantially. There was a sharp rise to a mean count of 65 CFUs/m² during donor and backtable preparation from a mean count of 5 CFUs/m². However, by the time the surgical excision of the allografts occurred, the numbers had dropped to a mean count of

25 CFUs/m². This is most likely due to the effective ventilation system within the OR. However, in contrast, with poor or no air ventilation systems within mortuaries and no restriction on staff numbers and multiple activities taking place, the chances of instrument contamination and hence a potential route of tissue contamination still remains high. Chosky *et al.* (1996) showed that covering instruments prior to use reduced bacterial contamination by four-fold, and they attributed half of the benefit to shorter instrument exposure time.

The results obtained here indicate that direct contamination of tissue with skin flora is high despite donor skin disinfection. As much as 46% of pairs of the same species recovered from tissue and donor's skin were shown to be related, with some having similarities above 95% (Table 11). Tissues contaminated with *E. coli* were retrieved from donors that had had a PM prior to the retrieval, which indicated superficial skin contamination with gut flora. Skin disinfection is directed against both resident and transient organisms (Burke *et al.* 1984) and all or most transient flora can normally be removed by this means. However, the skin disinfectants employed in this study may have been inefficient at removing *E. coli*, since identical strains were picked up from the donor's skin and donor's tissue, although bearing in mind that the *E.coli* could have disseminated to the tissue from the gut after death or after post-mortem.

Bacteria are situated within the hair follicles, deeper portions of the horny layer and the outer layers of the stratum (Kligman 1965). The mean microcolony size is of the order of 10⁴ viable cells per cm² of skin, and so the action of topical antibacterials will reduce the number of microbes in a microcolony without significantly reducing the number of microcolonies (Somerville & Noble 1973). Resident skin bacteria will therefore persist after skin disinfection. However, transient populations, which are acquired from the

environment are generally removed by washing, and in the case of the donor, organisms from the bowel such as *E. coli* contaminating the skin should also be effectively killed by the disinfectant.

To minimise tissue contamination, it is important that an antiseptic acts rapidly against a wide range of microorganisms. Staff are put under pressure to complete the retrieval within as short a time as possible, and so disinfection is achieved in a single application. In some cases the agent is not left to dry or left on for a period of time for the disinfectant to work against skin flora before incision commences. This could explain why *E. coli* was still found on the skin and then transferred to the graft. For effective skin disinfection, it is recommended that the antiseptic is applied twice thoroughly, paying particular attention around the thigh area of donors who have had a post mortem. The antiseptic must also be allowed to dry for it to be effective against skin flora (Kligman 1965). The use of isopropyl alcohol followed by iodine tincture is thought to be more effective as a skin disinfectant than the combination of chlorhexidine gluconate and isopropyl alcohol (Goldman *et al.* 1997).

2.4.2.1 Contribution of staff

Tissue contamination rates are thought to be governed by many factors such as donor selection, good sterile technique, use of sterile equipment and staff gowning up appropriately. In this study, staff were gowned similar to that of operating room staff, with surgical gown, head cap, face mask and sterile gloves (Fig. 6). These measures are considered effective in reducing bacterial air counts by preventing bacterial shedding.

Here, only staff skin flora rather than nasal or oral flora were compared with isolates recovered from the tissue, since skin flora is commonly shed onto clothes. Surgical face

masks have been worn by operating room staff for many decades to protect open wounds from potential bacterial contamination disseminated from the nose and mouth. Mitchell & Hunt (1991), however showed that a small number of mouth bacteria expelled by the surgeon and the scrubbed member of the surgical team during ordinary talking in a modern OR with forced ventilation system may contaminate the surgical wound, however not enough to cause an infection. It has been suggested that a face mask may even increase the dispersal of skin scales from the face by rubbing against the skin (Schweizer 1976). It is generally accepted that there is little evidence to support the use of masks in conventional theatres (Ayliffe 1991, Tunevall 1991, Mitchell & Hunt 1991). However, there is evidence that face masks, along with head cap and gown are essential at reducing air counts in OR with 'Vertical-laminar flow' ventilation system ('ultra-clean' theatre). Hubble *et al.* (1996) showed that in the absence of hat or masks there was a 22-fold increase in CFU on settle plates that were placed at waist height near the surgeon where the air flowed downwards from above the surgeon and then extracted at floor level.

The number of bacteria in air counts in mortuaries before and after retrieval varies considerably from one retrieval to another and cannot be readily compared with each other owing to the influence of other factors. Deijkers *et al.* (1997) found that the risk of contamination with low pathogenic organisms, mainly skin commensals, increased by a factor of 1.6 for each member added to the procurement team. However, this was not substantiated in the present study. The number of staff did not appear to have a significant affect on air counts (Table 6, cases 10, 14, 20) and even when the ventilation systems was switched off (Table 6: case 8). It is known that during moderate physical activity, individuals may shed approximately 1000 bacteria carrying skin scales per minute. The more active the person is, the more bacteria carrying particles are shed. In

cases 1, 4, 5, 7 and 17, multiple activities (PMs, embalming, etc) were taking place during retrievals, however, there were no significant changes in the air counts. Nevertheless conflicting results were obtained with cases 9 and 13 (Table 6). In both cases, the ventilation system was not switched on and there was an increase in the number of staff before and after, and also additional activities took place, yet the bacterial air count increased after the retrieval. This may have been due to increased skin shedding by an individual but this could not be confirmed.

2.4.3 Significance of the cause of death, time between death and retrieval and blood and bone marrow culture on bacterial contamination of tissue

Tissues from donors who have had a traumatic death are likely to be contaminated with pathogenic organisms. It has been suggested that tissues from donors with traumatic death are contaminated 3.4 times higher with organisms originating mainly from the gastrointestinal tract (Deijkers *et al.* 1997). The contamination of the donor's blood also increased by a factor of 14.6. Veen (1994) in his doctoral thesis showed that there is a relationship between cause of death and bacterial contamination of bone graft. He found that donors who had a traumatic death had a significantly higher number ($p < 0.0001$) of tissues contaminated with pathogenic organisms compared to donors with non-traumatic deaths. He also found that 16 of 17 (94%) donor's blood cultures (traumatic cause of death) were contaminated with species such as *Strep viridans*, *S. aureus*, *S. haemolyticus* and *E. coli*. In contrast, 6 of 63 (9.5%) donor's (non-traumatic) blood cultures were contaminated with only 2 types of organisms; *Strept viridans* & *S. aureus*. In this study, there were only 2 cases of donors that had died of traumatic causes. Donor 2 (Table 4) died of a road traffic accident, and did not have a post-mortem and neither were there any other activities taking place before or during the retrieval (Table 5). All of the tissues that were retrieved were found to be contaminated

(Table 7), and species isolated included *E. coli* (Table 8), which was also isolated from the donor's skin. Unfortunately, the relatedness between the two isolates by DNA fingerprinting was not performed (one of the isolates failed to grow after storage in glycerol broth). However, in other cases (6, 8 & 17), *E. coli* isolates recovered from tissue and donor were matched as identical by DNA fingerprinting. It is probable that the isolates in case 2 would also have proved to be related. Road traffic accident victims often sustain internal injuries, crushing, burns, laceration, severe cuts and open wounds in all parts of the body, which could result in spillage of bowel contents onto the donor's skin. This may explain why *E. coli* was recovered from case 2, even when no PM had been performed on donor or in the mortuary at the time.

Deijkers *et al.* (1997) recommended that donors with traumatic death should be excluded as they are most likely to have tissue that is contaminated with highly pathogenic organisms due to agonal bacteraemia (bacterial invasion of the blood with gut flora as a result of trauma or natural breakdown of barrier between bloodstream and gut flora leading to haematogenous spread to the bones. This was emphasised by the strong relationship between a positive blood culture and the contamination of bone with pathogenic organisms. Malinin *et al.* (2003) also found traumatic death such as drowning had a significant affect on contamination and in their case, *Clostridium* spp. were grown from the blood, marrow and tissue samples. At least one death has been reported, in a young man from clostridial infection following receipt of a soft tissue allograft. This finding had a huge impact on the American Tissue Banking community (Report of the Third World Congress on Tissue Banking and 26th Annual Meeting of the American Association of Tissue Bankers, August 23rd-27th, 2002, Boston) which led to urgent calls for increased vigilance with regards to presence of clostridia in transplanted tissues.

The time between donor's death and tissue retrieval is considered to be a risk factor associated with contamination. Malinin *et al.* (2003) took blood cultures from the vena cava via the saphenous vein to detect transmigration of microbes from the gut, and also bone marrow from the iliac crest prior to procurement. They found that it was more likely to find a positive blood or bone marrow culture than a positive tissue culture. The trend to positive results increased as the time from death increased, particularly after 12-18 h. They concluded that clostridial contamination was present in a significant number of tissue donors, particularly those with prolonged time between death and tissue excision. The study of Vehmeyer *et al.* (2002a) also demonstrated that the risk of blood contamination increased with time post mortem and the same increase was seen with organisms of high virulence. In donors with multiple trauma, the risk of blood contamination with organisms of high virulence was greater, but smaller in donors with preceding organ procurement. They suggested that blood cultures should be taken into account, since these can help to reveal contamination not detected by swab cultures.

In this study, the time between death and tissue retrieval varied from 7 h to 48 h in case 3. The average time between death and first incision was 16 h. The number of donors was too small to establish a significant relationship between time of death and tissue excision, and level of contamination, although it is noteworthy that in case 3 (Table 4), tissues were retrieved almost 48 h after death from a donor who had died traumatically and who had had a post mortem, and yet only 4 of the 6 tissues retrieved were found to be contaminated (Table 7) with swab cultures yielding CNS and a *Bacillus* spp. (Table 8).

Organisms may originate from i) the retrieval team; ii) the donor or iii) an endogenous source as a result of a clinically occult bacteraemia or by post mortem dissemination of microorganisms (Vehmeier *et al.* 1999, 2001). The results of bacteriological sampling alone are insufficient to identify the source of contamination which may have occurred during the retrieval process or due to transmigration of organisms pre or post-mortem.

Deijkers *et al.* (1997) considered that the presence of organisms of low pathogenicity was mainly associated with external sources, whereas highly pathogenic organisms were associated with endogenous sources, either from the bowel or clinical bacteraemia. Donor selection does not guarantee that the donor is free from clinical bacteraemia or agonal bacteria. Several studies have been carried out to link the bacteria recovered from the donor's blood with the retrieved tissue. Vehmeier *et al.* (1999) detected pathogenic bacteria in blood cultures from 8 of 95 donors that met the standard selection criteria issued by the EAMST and the EATB, both of which specify the exclusion of donors with signs of clinical infection. Some studies on blood cultures of donors have isolated organisms such as *Clostridium* spp. (Martinez *et al.* 1985, Malinin *et al.* 2003), *Streptococcus* spp. (Deijker *et al.* 1997) and *E. coli* and *Aeromonas* spp, (Vehmeier *et al.* 2001), all of which were also found on the tissue. In one study of 770 cadaver bone donors, positive blood cultures were found in 166, of which 45% had positive bone cultures. The range of pathogenic organisms isolated included *Streptococcus* spp. *S. aureus*, *Enterococcus* spp. *Clostridium* spp., and various coliforms (Martinez *et al.* 2003). Interestingly, almost all these species were recovered from bone specimens from donors who had negative blood or bone marrow cultures. However, the rate of tissue contamination varied considerably. Donors who had positive blood and bone marrow cultures had a higher rate of bacterial tissue cultures (48%), compared with donors with

negative blood and marrow cultures (7.3%) and from donors with only positive blood (15%) or marrow cultures (11%).

Blood or bone marrow cultures were not taken in this study, however organisms with potential pathogenicity were isolated from swab cultures of retrieved tissue. These included *S. aureus*, *E. coli*, *Bacillus* spp., *Aerococcus viridans*, *Branhamella* spp., and other Gram negative rods. With the exception of the first three groups of organisms, the remaining species were not recovered from the donor's skin or staff, indicating that contamination with *A. viridans* and/or, *Branhamella* spp. may have occurred via an endogenous or environmental source. *E. coli* is a natural resident of the bowel, and tissues from 5 different cases (Table 8: cases 2, 6, 8, 13, 17) yielded this species. *E. coli* was not recovered from the skin of donor 13 which may suggest endogenous or environmental contamination. However, in case 2, *E. coli* was recovered from the retrieved tissue and the skin of the donor who did not have a PM prior to the retrieval, which possibly indicates environmental contamination. Cases 6, 8, and 17 (Table 4) had all had PMs and there was a 100% match between the DNA of tissue and donor's skin isolates. Therefore, even with the lack of blood or bone marrow cultures, the widespread presence of coliform organisms supports the likelihood of endogenous contamination of retrieved tissue.

Most of the potentially pathogenic pathogens were isolated from femoral heads and shafts, with the exception of *E. coli* (Table 9), which was retrieved from all types of tissue recovered, indicating that femoral heads and shafts are most likely to be contaminated with pathogenic organisms due to their close proximity to the abdomen.

To date, no other studies linking isolates from the donor and the environment to the contaminant of retrieved tissue by DNA fingerprinting have been reported.

2.4.4 RAPD and limitations

The RAPD method was first described in the mid 1980s as a rapid means of identifying genetic relationships between bacteria. The technique generates polymorphic band patterns from the genomic DNA of a given organism amplified by PCR using arbitrary DNA sequence primers.

If total DNA is used, RAPD yields abundant information about the analysed genome in a rapid and inexpensive way. Different primers bind to complementary priming regions of the genomic DNA, and minor variations in the DNA sequences of different isolates lead to distinct fingerprinting patterns that are discriminatory. This information may be used in various types of genetic studies, such as hybrid detection, intra and interspecific genetic variation, genetic identity establishment, clonal variation analysis and, when combined with other methods, the data are useful for the elaboration of genetic maps.

The major advantages of RAPD assays over other DNA typing method are 1) prior knowledge of the sequence of the organism is not required; 2) primers are chosen arbitrarily, and in practice the method can be applied to any species; 3) crudely extracted DNA is sufficient for most assays and the method of extraction is similar for most species. However for some organisms such as *C. albicans* and *Serratia marcescens* purified DNA is required for optimal results. Other advantages are: the method can be completed in a day and the same primer sequence can be used for different species i.e., ERIC, M13, etc (Towner and Grundmann 2001, Dassanayake and Samaranayake 2003).

However, there are limitations to the RAPD procedure. The clarity of the product and the complexity of fingerprints depends greatly on technical protocols followed. There

should be strict control of technical parameters such as the quality and quantity of the reagents used, the primer-to-template concentration ratio, the quality of the *Taq* polymerase, the PCR buffer concentration and the magnesium concentration in the reaction mixture. It also vital that no contaminants are introduced during template preparation as this may lead to non-specific amplicons and misinterpretations of results. All these factors have been cited to influence the reproducibility of the technique (Ellsworth *et al.* 1993, Meunier & Grimont 1993, Tyler *et al.* 1997). Theoretically, the specificity of the amplification process depends on primer-template interactions, and so changes in the concentration of template DNA or primer will affect the size of the PCR products amplified, and hence result in different RAPD fingerprints. Nevertheless, if a standardised DNA extraction method is used with cell suspensions of broadly equivalent initial cell densities, reproducible fingerprints can be obtained with a range of bacterial species without precise measurement of the template DNA concentration (Penner *et al.* 1993, Towner & Grundmann 2001). Moreover, due to the anonymous character of polymorphic bands and the difficulties for establishing homologies, it is recommended that RAPD be used only to compare isolates within a species in a defined incident as DNA profiles of organisms from completely different sources may be similar. Tyler *et al.* (1997) recommended that RAPD should only be applied for immediate studies, and standard conditions should not be applied to large scale projects where past results are compared or collated with present or future ones. Therefore, RAPD is at its most useful when profiles are used to distinguish between strains rather than to confirm their identity.

The introduction of RAPD analysis beads, which are supplied individually in a quality controlled, premixed, room temperature-stable format and optimised for RAPD reactions, have eliminated or reduced many of the possible factors that influence

variation in DNA fingerprints. Each bead contains *AmpliTaq* DNA polymerase, buffer and magnesium concentration, nucleotides, etc, and so allows standardisation of reagents.

The RAPD method was particularly useful for this study since only two types of primers were used for 12 different species and it was possible to process a large number of isolates over a short period of time. Isolates of the same species recovered from different sources and tissue within each retrieval underwent the RAPD assay. The banding profiles were in most cases discrete, and could be automatically identified and digitized by a computer-assisted system (Gel Compar). The fingerprints were analyzed quantitatively, by taking into consideration the presence or absence of bands, their intensity, and the position of each band. The matrix of similarity coefficients was then used to generate a phylogenetic tree (dendrogram) by the unweighted pair-group clustering method using arithmetic averages (UPGMA), (Dassanayake & Samaranayake 2003). The outcome of the analysis is expressed in terms of percentage similarity between each individual pair of isolates and in keeping with many published studies, a cut-off point of 85% relatedness was used to denote genetically related strains. Further detail on gel analysis is given in Appendix 2.

Chapter 3

The validation of current and alternative sterility testing methods for estimating the bacterial load of surgically retrieved bone

3.1 Introduction

Tissue banks are expanding with time, ranging from hospital based bone banks to multi-organ tissue banks run by the NBA. Tissue banks operated by the NBS and independent tissue banks follow recommendations of the British Orthopaedic Association (BOA). These stipulate that bone banking should be organised in a similar fashion to blood banking to ensure similar levels of safety and security, and confirm that clinical tissues should be free from viral and bacterial contamination. The BATB and the CMSBTT have set standards and guidance for the selection of donors, serological testing, processing and storage of human tissues to minimise the risk of transmitting disease.

The number of fresh frozen femoral heads issued nationally (all NBS tissue banks) without any further processing on the basis of negative bacterial contamination in 2003/2004 was 1612. Organisations that bank fresh frozen femoral heads employ methods to assess bacterial bioburden at the time of procurement. However, no standard technique to assess bacterial contamination has yet been published. The use of human allograft tissues have increased dramatically in recent years, and with this increase has come a greater reliance on the use of culturing bone chip and swab culturing techniques to assess for microbial contamination prior to distribution. A survey carried out by the Working Party of CMSBTT & NIBSC the Red Book Committee (1993) showed that the techniques used for bacterial sampling, the type of culture medium and incubation period varies considerably from one tissue bank to another. Examples of methods used are given in Table 13.

Table 13. Types of methods used for bioburden assessment

Bacterial Sampling	Culture Medium	Incubation Period
Swab and bone chip	Tryptone soya broth	24h
Bone chip only	Thioglycollate broth	48h
Bone chip and wash culture	Robertson's broth medium	5 days
	Wilkins Chalgren's broth	7 days
	Blood agar	14 days

Swab and bone chip culture is performed by the majority of tissue banks which rely on these results before the tissues can be released. The surface of the bone area is swabbed with a cotton swab just after procurement or prior to processing. For bone chip culture, a small fragment of the bone is cut and placed in broth, which is incubated for 24 h up to 14 days.

Due to the wide variety of techniques employed for bioburden assessment, the reported percentage of retrieved heads contaminated with bacteria ranges from less than 5% at one centre to 50% (Figure 15) at another and therefore there is no consensus of the relevance of the results obtained (data obtained from questionnaire sent to Tissue Banks). It has also been reported in the literature (Veen *et al.* 1994) that swabs have limitations, both in sensitivity and reproducibility, so their suitability as a final sterility release method is not ideal.

Where contamination is found, the policies of different banks relating to the use of the tissue also varies considerably. Surgically retrieved bones that are found to be free of contamination at the time of procurement are usually issued to surgeons as fresh frozen femoral heads without any further processing or terminal sterilisation. In contrast, if

bone chip and swab culture yielded bacteria, some banks choose to discard the bone whereas others that have the facility for processing and terminal sterilisation will retain them for further treatment. It follows that bone tissues are issued without processing and sterilisation on the basis of negative bacterial contamination, it is therefore vital that the sampling method used is stringent and sensitive so that false positive, and more importantly false negative results are not given, which may lead to post-operative infections in recipients. Crawford *et al.* (2005) has shown that the use of sterile allograft appears to be associated with a significant reduction in the risk of post-operative infection as compared to just aseptic graft. However, they do state that larger clinical studies are necessary to confirm this observation.

The method employed to assess bacterial contamination should therefore be rapid, qualitative, quantitative and efficient at detecting contamination. Little or no research has been carried out to assess the efficiency of techniques used for sampling in terms of recovery of a range of different organisms (Veen 1994).

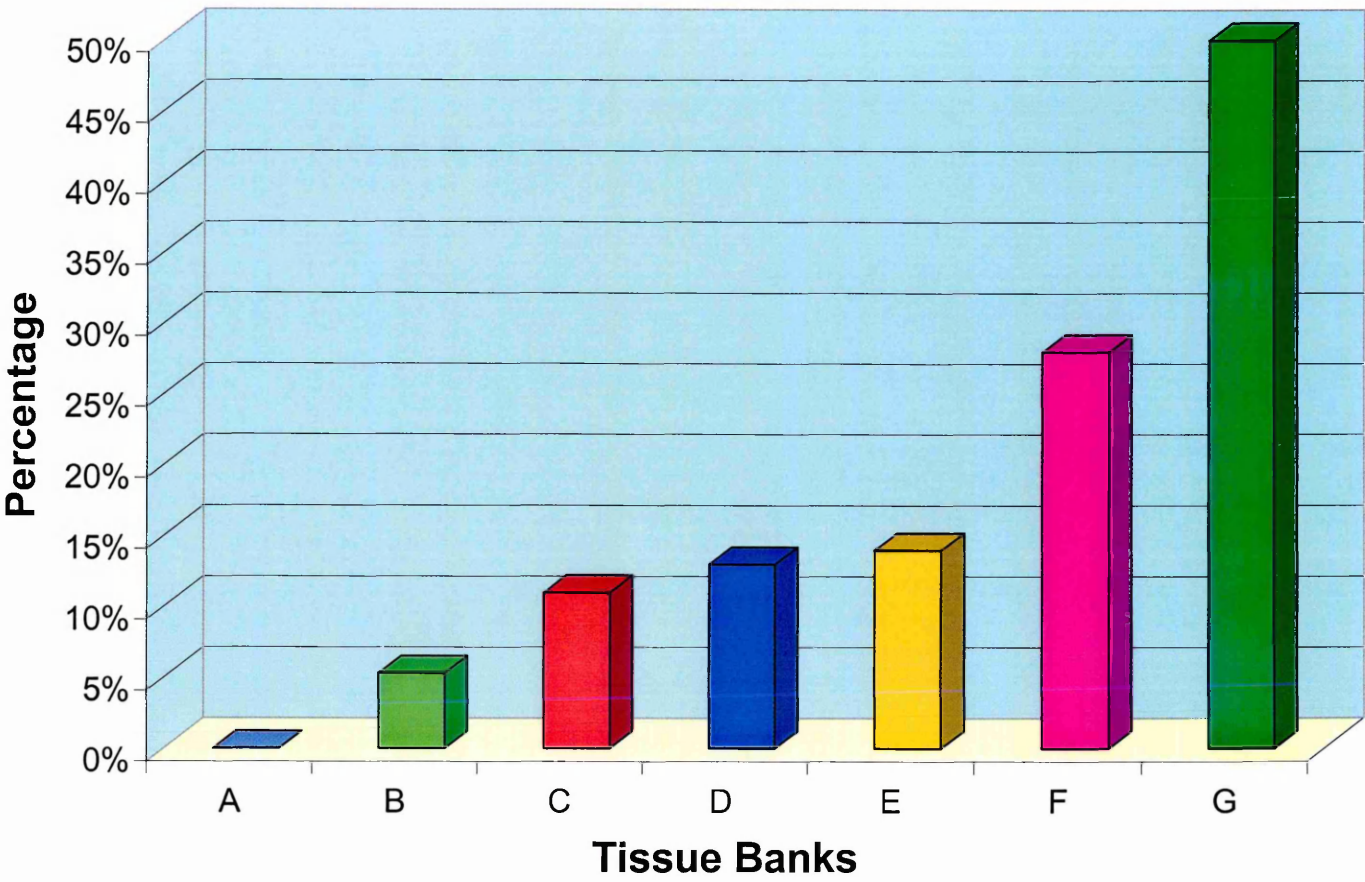


Figure. 15 Percentage of contaminated surgically retrieved femoral heads from seven different tissue banks.

- A: Cambridge**
- B: Newcastle**
- C: Glasgow and West of Scotland**
- D: North London**
- E: Mersey and North Wales Oswestry**
- F: Inverness**

3.2 Material and methods

In order to evaluate the efficiency of a sterility testing method, it was necessary to establish a reproducible measurement of the bacterial load on a tissue. This was performed by spiking bone with suspensions of bacterial species of known concentration and determining the efficacy of the sterility method by recovering growth with selective agars for each species.

3.2.1 Cocktail of strains used for spiking

Two cocktail panels of strains were used.

3.2.1.1 Panel 1

This panel consisted of *Enterococcus faecalis* NCTC 775, *Pseudomonas aeruginosa* NCTC 6749, and *Escherichia coli* supplied by Laboratory of Enteric Pathogens, Centre for Infections, Health Protection Agency (CFI, HPA). The *E. faecalis* strain was grown in 10 ml of Brain Heart Infusion broth (BHI), and *P. aeruginosa* and *E. coli* strains were grown in 10 ml of Nutrient broth (NB) at 37 °C, overnight. The concentration of *E. faecalis* and *E. coli* corresponded to approximately 5×10^7 colony forming units (CFU) per ml, and for *P. aeruginosa* to approximately 1×10^8 CFU per ml.

Overnight broth cultures of *E. faecalis*, *E. coli* and *P. aeruginosa* were combined in a ratio of 2:2:1, respectively (see A). Serial 10-fold dilutions of the cocktail were made in 9 ml of 1/4 strength Ringers solution to obtain the desired inoculum in CFU per 0.1ml (see B), which was confirmed by plating on a selective medium.

A:

	ratio	
<i>E. faecalis</i>	2	2×10^7 in 0.4 ml
<i>E. coli</i>	2	2×10^7 in 0.4 ml
<i>Pseudomonas</i>	1	2×10^7 in 0.2 ml

B:

Average count of each species per 0.1ml of the cocktail were as follows:

<i>E. faecalis</i>	2362 CFUs
<i>E. coli</i>	2946 CFUs
<i>Pseudomonas</i>	2025 CFUs

Counts were taken each time a tissue was spiked.

3.2.1.2 Panel 2:

This consisted of *Staphylococcus saprophyticus* NCTC 689/96, Methicillin resistant *Staphylococcus aureus*, CPHL ST96 (MRSA) and *Micrococcus luteus*, NCTC 7495.

The *S. saprophyticus* and MRSA strains were grown in 10 ml NB overnight at 37 °C.

The turbidity of the two strains reached approximately 10^8 CFU /ml. The *M. luteus* strain was grown on BHI agar at 37 °C for 48 h. The growth was harvested in PBS solution and serially diluted in ¼ strength Ringers solution until the desired inoculum in CFU per 0.1 ml was reached similar to that of *S. saprophyticus* and MRSA. The three strains were combined in equal ratio and serially diluted to give the desired CFU per 0.1ml (10^4), which was confirmed by plating on selective medium. *S. saprophyticus* was chosen because of the ease of separation from amongst the other species using selective medium.

3.2.2 Media:

Selective media was used to isolate each type of species from the cocktail.

3.2.2.1 Panel 1 selective media:

Brain heart infusion agar (BHI) contained staph/strep supplement (nalidixic acid 15 mg/l and colistin sulphate 10 mg/l) for the isolation of *E. faecalis*. *E. coli* was recovered on Nutrient agar (NA) containing 32 mg/l apramycin (NAA). *P. aeruginosa* was selected on NA containing cephaloridine (32 mg/l), fucidin (32 mg/l), and 32 mg/l of cefrimide (CFC).

3.2.2.2 Panel 2 selective media

Nutrient agar with 32 mg/l of nitrofurantoin was used for isolation of *M. luteus*, and with 2 mg/l novobiocin for isolation of *S. saprophyticus*. MRSA were recovered on mannitol salt agar with oxacillin (2 mg/l).

3.2.3 Tissue

Tissues that were unsuitable for clinical use were used for this study. This included femoral heads from patients that had undergone hip replacement and femoral heads from cadavers. Each femoral head was allocated a unique serial number and stored at -70°C and when required was defrosted overnight in the refrigerator. On average, femoral heads weighed 102 g and the approximate area was 6 cm by 6 cm (height and width).

3.2.4 Spiking of femoral heads with bacteria

During spiking and adhesion of bacteria on tissue, dehydration can occur, and so a pilot study was carried out (appendix 6.2.1) to determine if there was a reduction in the number of CFU over a period of 48 h of dehydration for species used for spiking. The study showed that no *P. aeruginosa* colonies were recovered at 24 h whereas in contrast, there was no reduction in the viable count for *M. luteus* (Table 16 and Fig. 23). The pilot study also showed that after 30 min of dehydration the viable count was only reduced 2-fold. As a result the cocktail panels were allowed 15 min adhesion time, and dehydration was reduced by keeping the tissue in a container partially covered with a lid.

In total, 100 femoral heads were used in the spiking experiment. The same femoral heads were used for filter culture and wash culture technique (25 femoral heads for panel 1 and 25 for panel 2). Separate femoral heads were used for the swab culture technique (25 femoral heads for panel 1 and 25 for panel 2). Each sample tissue was maintained in a sterile plastic pot and a section of the bone (cartilage area), approximately 9 cm² area of the tissue was inoculated with 0.1 ml of bacterial suspension containing about 3000 CFU (Fig. 16). Each cocktail was tested in pairs for a) recovery by washing and b) recovery by swabbing. For recovery by swab, femoral heads were spiked in 1 cm² areas until a total of 9 cm² area was covered and 15 min adhesion time was allowed.

Aliquots of each cocktail were plated out on selective medium to determine the original count (CFU) for each species before recovery. This was repeated for each femoral head that as spiked.

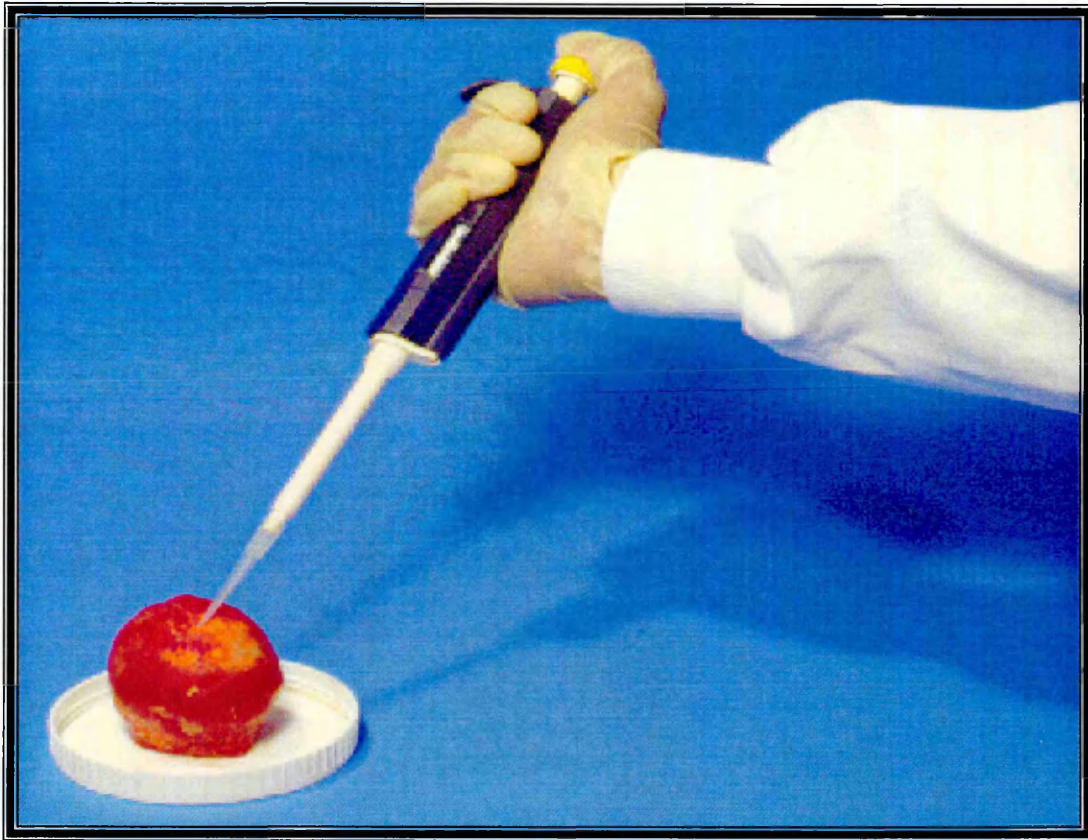


Figure 16. Spiking femoral head with a cocktail of bacteria

3.2.5 Recovery

3.2.5.1 Washing

The spiked bone was placed in a sterile stomacher bag (approx A4 in size) containing an inner mesh bag with pore size of $< 0.5\text{mm}$ in diameter to retain small particles (Fig. 17). To the bag was added 350 ml of sterile phosphate buffer (PBS) with 0.2% Triton and this was shaken vigorously for 3 min on a mechanical shaker at room temperature.

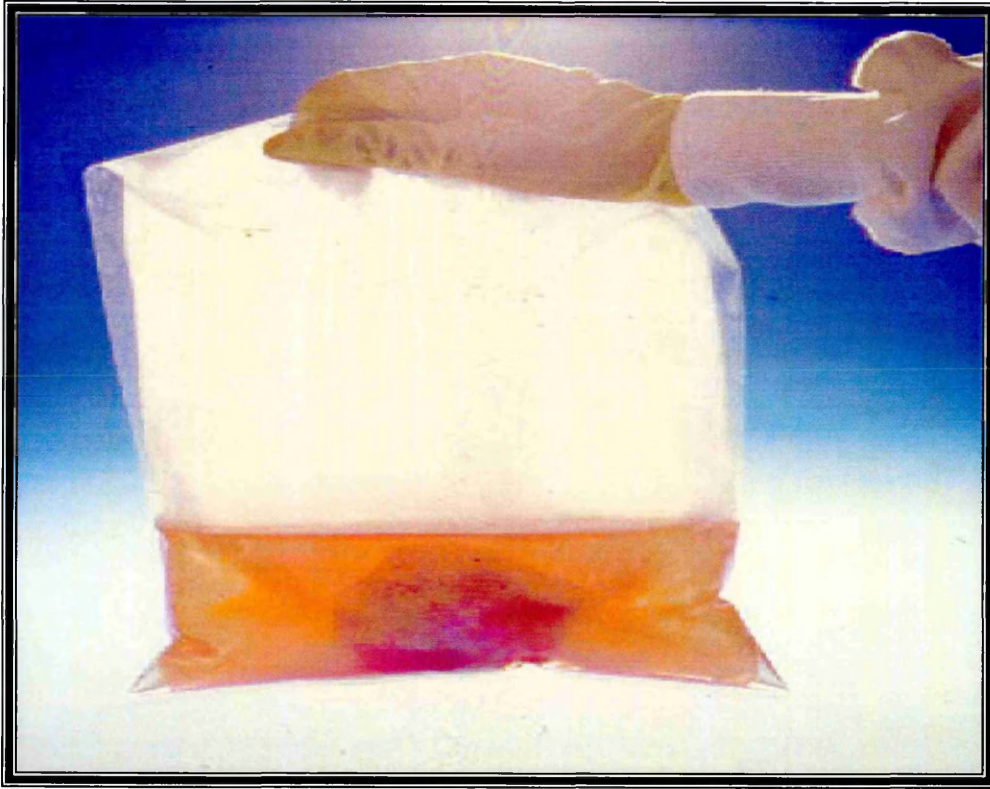


Figure 17. A stomacher bag containing a spiked femoral head and wash solution

Aliquots (0.2 ml) of the wash solution were also directly inoculated on the selective media for each species using an automatic sterile pipette to compare the relative recovery rates by filtration and direct plating of wash fluid. The plates were incubated at 37°C for 48 h and the CFU were counted.

3.2.5.2 Filtration

Three times 100-ml aliquots of the 350 ml wash solution were vacuum filtered on separate sterile 37 mm diameter membrane filters of 0.45- μ m pore size (Sartorius), with two top layer glass fibre pre-filters to remove large particles and to prevent blocking of the filter. The membranes were recovered and placed on each selective medium with the contact side (grid) up. Three aliquots were taken for each selective medium used to recover the members of each panel of species. After 48 h incubation at 37°C, the

number of CFU recovered was recorded for each species. The total numbers of CFU recovered from both panels were then compared to the original inoculum level to estimate the percentage recovery for each species.

3.2.5.3 Swab culture technique

The surface of a femoral head was swabbed with a sterile cotton swab moistened with 0.2 % Triton (Fig. 18). The cotton swab was rubbed and rotated five times across the surface of the tissue, and then placed into 3.5 ml of Triton. The swab was vortexed for 1 min to release bacterial cells from the cotton swab and 1 ml aliquots of the fluid were plated over the entire well-dried selective agar media using a sterile loop and incubated at 37°C. The total number of CFU recovered was counted after 24 h incubation for panel 1 species and 48 h incubation of panel 2 species.

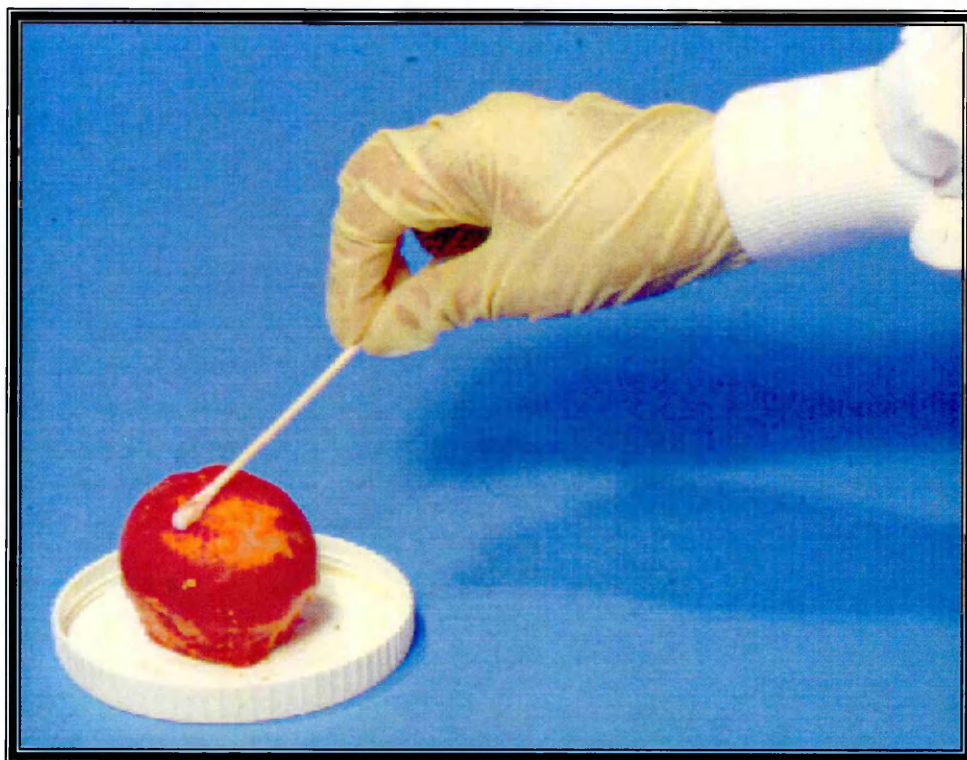


Figure 18. Femoral head swabbed with cotton swab.

3.2.6 Statistical analysis:

Pairwise non-parametric tests for differences in mean rate of recovery were used to determine significant differences between the methods used. Wilcoxon signed rank test was used for filter vs. wash tests, as these methods were carried out on the same physical samples (matched pairs). The Wilcoxon rank sum (Mann-Whitney) test was used for filter vs. swab and swab vs. wash tests, as they were performed on different physical samples. Advice on statistical methods was given by the Statistic Dept, Centre for Infections, Colindale.

3.3 Results

As detailed in the methods, bioburden recovery was estimated by seeding the surface of the femoral heads with the different cocktails of bacterial species. Bacteria were then recovered by one of three methods: swab culture, filtration and wash culture.

The mean percentage recovery (inc. standard error bars) of bioburden from femoral heads with the three techniques are presented in Figure 19. For the filtering technique, percentage recoveries ranged from 48% to 289%, and for the swab culture from 4% to 18%. There was a significant difference between the two methods ($P < 0.05$) indicating that the filter culture technique was more effective at recovering bioburden. However, bioburden recovery with this filter technique was not consistent because the standard error for *M. luteus* was 150.21 (degrees of freedom 27.013). In contrast, the swab culture technique gave more consistent results despite being less effective at recovering bioburden. The mean percentage of bioburden recovered using the wash technique followed by direct inoculation of the wash ranged from 0% to 0.08%, and hence the reason for no visible bars shown in Fig. 19. There was a significant difference between the inoculation of the wash culture and swab culture technique ($P \leq 0.05$) suggesting that wash culture technique is inefficient at detecting bioburden.

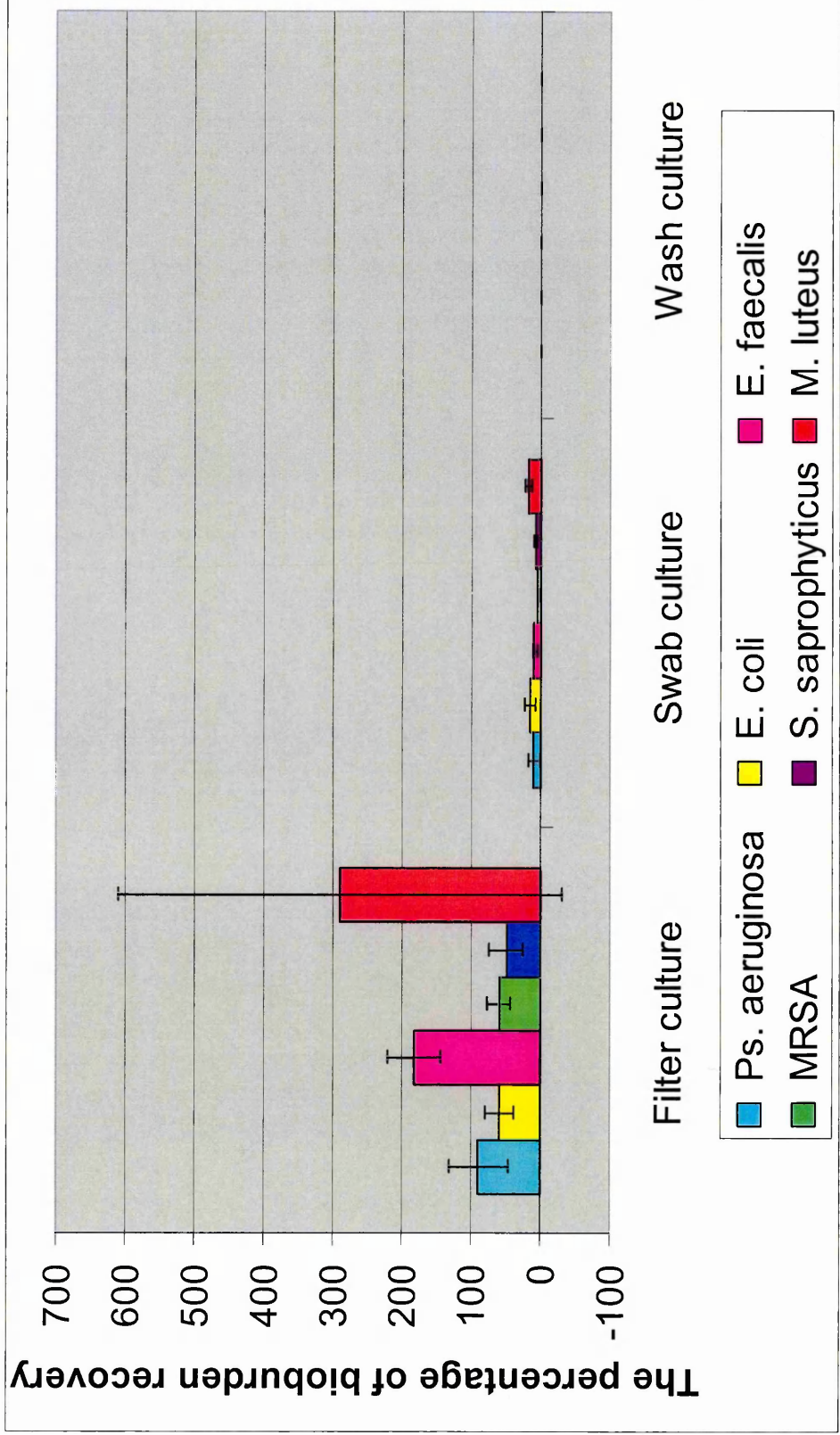


Figure 19. The percentage of bioburden recovered from spiked femoral heads.

3.4 Discussion and conclusion

Washing femoral heads with mild detergent is purported to remove bacteria from the surface of the tissues effectively, thereby allowing the bioburden to be assessed quantitatively and qualitatively. However culturing small aliquots such as 200µl of 500 ml (0.04% of the total wash volume) is clearly insensitive as only those tissues that are heavily contaminated with bacteria i.e., ($>10^3$ in 100 ml) would yield positive growth. This means that of 100 CFU, only 4 bacterial cells would be sampled in a 200 ml aliquot. This method is therefore inadequate for assessing the bioburden of the retrieved tissue. In one particular tissue bank (Fig. 15, Tissue Bank A, Cambridge) femoral head bioburdens were assessed by culturing 200 µl of 500 ml 0.9% sodium chloride wash onto different types of medium (blood agar and Sabouraud's agar) incubated in different conditions. This may explain why this tissue bank reported the least number of contaminated femoral heads compared to other banks. The conventional swab culture technique also lacks the sensitivity achieved by the filtered wash culture.

Washing bone with a mild detergent, followed by culturing the filtered wash can be readily adopted as a routine bioburden test to provide reliable and valid results. The contribution of endogenous contamination is likely to be very small and as the tissue is retrieved under operating theatre conditions, contamination from exogenous sources should be minimal. By the cotton swab technique it was estimated to sample approximately $\frac{1}{4}$ of the surface of the femoral head. The percentage recovery (which ranged from 4 to 18% in this study) suggest that this was representative of the original inoculum since only a small portion of the surface was spiked (9 cm² area) and so swabbing appeared to be an efficient means of sampling the bone surface and would be most applicable for femoral heads with relatively heavy contamination. The filter

technique was by far the most efficient way of sampling bioburden although it was technically demanding, labour intensive and highly variable for two of the species used. Counts for *M. luteus* and *E. faecalis* were markedly increased, and a recovery of above 100% was observed (Fig. 19). This increase was probably due to the presence of the detergent, which may have dispersed the bacterial aggregates to more discrete colony forming units and hence increased the counts (personel communication, Peter Hoffman). *Micrococcus* spp. tend to grow in large clusters when grown in solid medium or in broth. A mild detergent like Triton or Tween x100 can break up microcolonies or clusters quite effectively (Williamson and Kligman 1965) giving rise to a higher CFU count. This also could apply to *E. faecalis*.

3.4.1 Sensitivity of swab cultures

The results from this study have shown that swab culture and direct culturing of bone wash give a poor yield of positive cultures. Therefore, currently there is a significant underestimation of detection rates of allografts in Tissue Banks that use the two methods for assessing contamination. This has implications for the recipients of bone from those banks, particularly when the allograft material is not secondarily sterilized such as the case with fresh frozen osteochondral allografts. This is important given increasing allograft usage and the rising numbers of revision joint arthroplasty and impaction grafting procedures being performed.

The detection rates of contamination would be higher if a more sensitive sterility testing method is used such as culturing the entire tissue into broth, as recommended by a number of authors (Veen *et al.* 1994 and Vehmeyer *et al.* 2001 & 2002b). Veen *et al.* (1994) found that 92% of the tissues were contaminated when the whole tissue was cultured in broth but the positivity rates for the swab culture technique was 9% (culturing swab into dry agar) and 36% (swab in broth). Vehmeyer *et al.* (2001)

compared the results of blood and swab cultures of two post mortem bone donors with procured grafts which were cultured in entirety. In one donor who died of drowning, three of the entire graft cultures were positive with the same organisms of high pathogenicity as the blood cultures, whilst the swab culture of only one graft was positive. All of the six grafts that were swab culture negative were found to be contaminated following culture of the entire graft (Table 14). In the second donor, who died from myocardial infarction, four entire cultures were positive with the same organisms of high pathogenicity as the blood culture, whilst the swab cultures of three grafts were positive. These results confirm the limited sensitivity of swab culturing technique as microorganisms inside a graft that have been disseminated through the blood stream may also remain undetected.

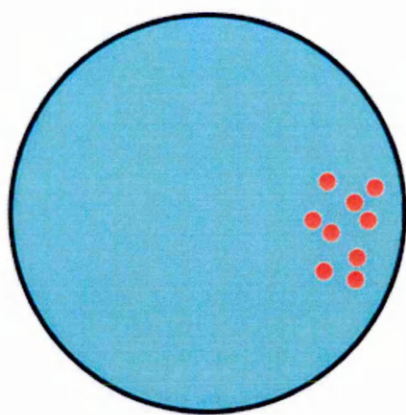
Table 14. Results of the swab cultures and the cultures of the entire graft from a donor who died of drowning (Vehmeier *et al.* 2001).

Graft	Swab culture	Culture of entire
Femur proximal left	No microorganisms	CNS
Femur distal left	No microorganisms	CNS
Tibia proximal left	No microorganisms	<i>Staphylococcus</i>
Fibula proximal left	CNS	CNS
Achilles tendon left	No microorganisms	Not cultured
Fascia lata left	No microorganisms	CNS
Femur proximal	<i>Aeromonas</i> spp.	CNS
Femur distal right	CNS	<i>Aeromonas</i> spp.
Tibia proximal right	No microorganisms	<i>Aeromonas</i> spp.
Fibula proximal	CNS	CNS
Achilles tendon right	CNS	<i>S. aureus</i>
Hemipelvis right	No microorganisms	CNS

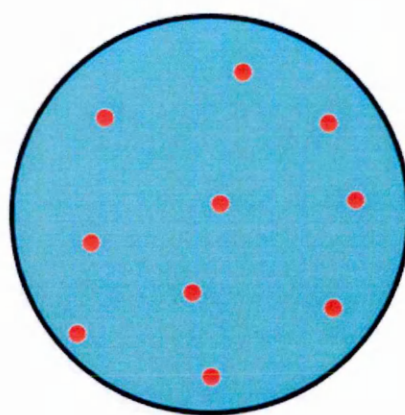
A review of the microbiological monitoring of bone grafts at Cambridge Tissue Bank (Farrington *et al.* 1998) revealed that contamination of bone on receipt by the bank decreased during a two year study and suggested that this may have been due to the increasing experience of staff harvesting bone. The methods used by this bank for microbiological monitoring before processing were culturing bone chips into enrichment broth and direct culture of wash. My study has shown that direct culturing of wash culture was inadequate at detecting contamination whereas only 0.08% of the bioburden applied experimentally was recovered from spiked tissue, even with the use of a mild detergent. Indeed, the poor recovery of bioburden by the wash culture method may give a sense of false security that procurement methods were improving. Grafts

that were negative by swab culture could in fact be found to be contaminated if cultured entirely into broth. Vehmeyer *et al.* (2002b) analyzed the bacterial contamination rate of femoral head allografts from living donors and determined the true bacterial load with cultures from the grafts in their entirety, and found that 9% (10 out of 91) of the grafts that were initially swab culture negative yielded normal skin contaminants after they were cultured in entirety in a specially prepared medium. Culturing the entire graft into broth however, would not be practical, as the intended tissue would no longer be suitable for surgical use. Culturing the filtered wash would be more suitable for detecting contamination as it ensures that the entire surface of the tissue is sampled without affecting the intended use of the tissue.

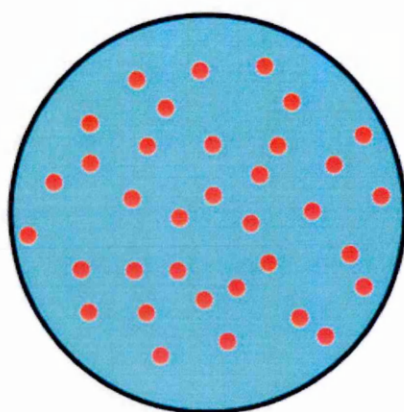
Tissue may be contaminated in a variety of ways as shown in Fig. 20. If the tissue has heavy, localized contamination (Fig. 20A), the chance of detecting any surface bacteria by swab culture would be minimal, unless the entire surface of the tissue is thoroughly swabbed. The chance of missing bacteria remains the same even when tissue is contaminated with low levels of bioburden, spread out evenly over the surface of the tissue. In this study, the swab culture technique only recovered an average maximum of 18% of the bioburden from the tissue despite being spiked with at least 3000 CFUs. This level of contamination would exceed the representation shown in Fig. 20 C. In all three types of contamination (A, B, and C), washing the bone followed by culturing the filtered wash will detect contamination and the graft tissue will not be affected, and there would be a reduced sampling error, in contrast to swabbing. The only advantage the swab culture technique gives is that it may yield more consistent results than filtration despite being less effective at recovering bioburden.



A: heavy, localized contamination



B: light, evenly spread contamination



C: heavy, evenly spread contamination

Figure 20: Diagrammatic representation of the distribution of bacteria on the tissue surface.

Quantitative studies on the swab culture technique have been carried out by Nystrom (1978) and Ronholdt and Bogdanský (2005). Nystrom (1978) looked at three different ways cotton swabs could be handled, and the percentage that was recovered by each technique. The three methods used were i) the transfer of staphylococci with a swab from an inoculated to a non-inoculated agar culture plate, ii) rinsing of the swab in a small volume of saline and culturing rinse fluid, iii) the swab was kept in Stuart's transport medium, as is the usual procedure in clinical practice, and then plated onto agar medium. The percentages that were recovered were, 19%, 39% and 4% respectively. The author concluded that culturing the swab rinse fluid was the most efficient for a quantitative bioburden assessment. Ronholdt & Bogdanský (2005) quantitative investigations of the swab sampling system also showed that the method exhibited low sensitivity, and recovery was highly variable from spiked allograft tissue.

In my study, prior to the investigation on the recovery on bioburden, a pilot study (Appendix 6.2.3) was carried out to find out how many CFUs could be recovered from an inoculated swab. This also revealed that not all the CFUs are recovered from the swab, on average 71% of *E. faecalis*, 23% of *P. aeruginosa* and 56% *E. coli* CFUs were recovered. Taken together with this investigation the studies of Nystrom (1978), and Ronholdt & Bogdanský (2005), the consensus is that swab cultures methods used for bioburden assessment pre and post-processing for tissue that are destined for use as fresh frozen grafts (i.e. osteochondral grafts), must sample as much of the tissue area as possible. This should be then directly inoculated into enrichment broth and subsequently subcultured on selective medium. For any quantitative assessment or tissue that is destined as a fresh frozen allograft, washing the entire graft in a mild detergent and subsequently culturing the filtered wash would be the most ideal method.

Chapter 4

Development of protocols for decontamination of
Achilles and Patella tendon without the use of
gamma irradiation and ethylene oxide

4.1 Introduction

The knee comprises the joint between the femur and the tibia, but also the joint between the patella and the front of the femur. Between the femur and the tibia sit two crescentic cartilage or menisci. These fibro-cartilaginous disc dissipate the compressive forces between across the knee and thereby avoid excessive loading, wear and damage. The ligments around the knee stabilise the knee. They include the collateral ligaments; medial and lateral, lying either side of the knee and the cruciate ligaments, anterior (ACL) and posterior cruciate ligament (PCL) lying within the joint (Fig. 21).

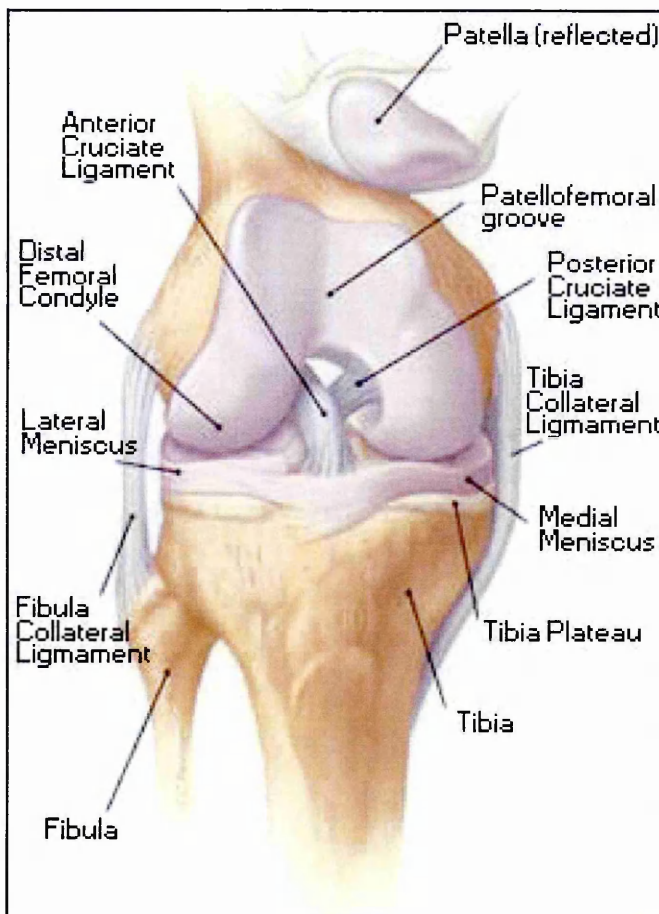


Figure 21. Knee showing anterior and posterior cruciate ligament
www.orthopaedics.co.uk/boc/patients/anterior-cruciate-indications.htm.

Anterior or Posterior Cruciate Ligaments (ACL, PCL) reconstruction involves the replacement of the injured ACL with a tendon graft procured from one of two sources: autografts, which are obtained intraoperatively from the patient undergoing the reconstructive procedure, and allografts, which are recovered from cadaveric donation (Crawford *et al.* 2005). The aim of reconstruction of the ACL is to restore stability of the knee without restricting its other functions especially motion. The use of cadaveric Patellar and Achilles tendon allografts (Fig. 3) to replace ACL is considered to be a life enhancing procedure rather than life-saving. Strict measures are taken to prevent disease transmission that may cause morbidity or mortality following allograft transplantation. However, infections do still occur which have led to morbidity and mortality such as clostridial infections (Barbour & King 2003, Malanin *et al.* 2003, Kainer *et al.* 2004). Several issues related to tissue transplantation safety have been highlighted by Crawford *et al.* (2005). In their study, infections did not occur among recipients of autografts or allograft tendons that had undergone a sterilization process; whereas the infection rate among recipients of allografts that were not sterilized was 4.4%. Species that were isolated from post-operative wounds included *S. aureus* and other staphylococci, *Enterococcus faecalis*, *Serratia liquefaciens* and various other Gram negative rods, and yeasts from post-operative wounds. Data from the National Bacteriology Laboratory, Colindale (between 2003 and 2004) for species recovered from tendons (NBS, Amanda Ranson) confirm the prevalence of bacteria such as staphylococci, diphtheroids, acinetobacters with the occasional coliform, fastidious Gram negative species and clostridia. If sterilisation of tendons carrying these types of organisms is not achieved, infection following transplantation could possibly occur. Kainer *et al.* 2004 highlighted several factors that contributed to clostridial infection, one of which was implanted tissue were not processed with the use of methods that achieved sterility or that were sporicidal, or there could have been a false negative as a

result of carry over of antimicrobial solution.

Tissue banks obtain allografts after careful donor screening and aseptic retrieval. The allografts are processed and decontaminated to minimise bacterial and fungal contamination. Some tissue banks remain concerned that allografts processed in this way may still harbour viruses such as HIV (Buck *et al.* 1989), and therefore they choose to terminally sterilise tendons using either gamma irradiation or ethylene oxide (EtOx) to ensure sterility, irrespective of the post decontamination bacteriology results. Both types of terminal sterilisation are said to be effective against bacteria and viruses (Jordy *et al.* 1975, Mermel *et al.* 1994). The effectiveness is further supported by a study carried out by Pruss *et al.* (2002) who has quantified the affect of gamma irradiation on human cortical bone transplants contaminated with enveloped viruses (HIV type 2, hepatitis A virus and polio virus and other model viruses such as Bovine parovirus for parovirus B19. They had found that a dose of approximately 34 kGy was necessary to achieve a reduction infectivity titre of 4 log₁₀. For effective sterilisation of frozen bone transplants, a dose of 34 kGy is recommended. However, the LSTS gamma irradiate tendons only if bacterial sterility testing failed following decontamination.

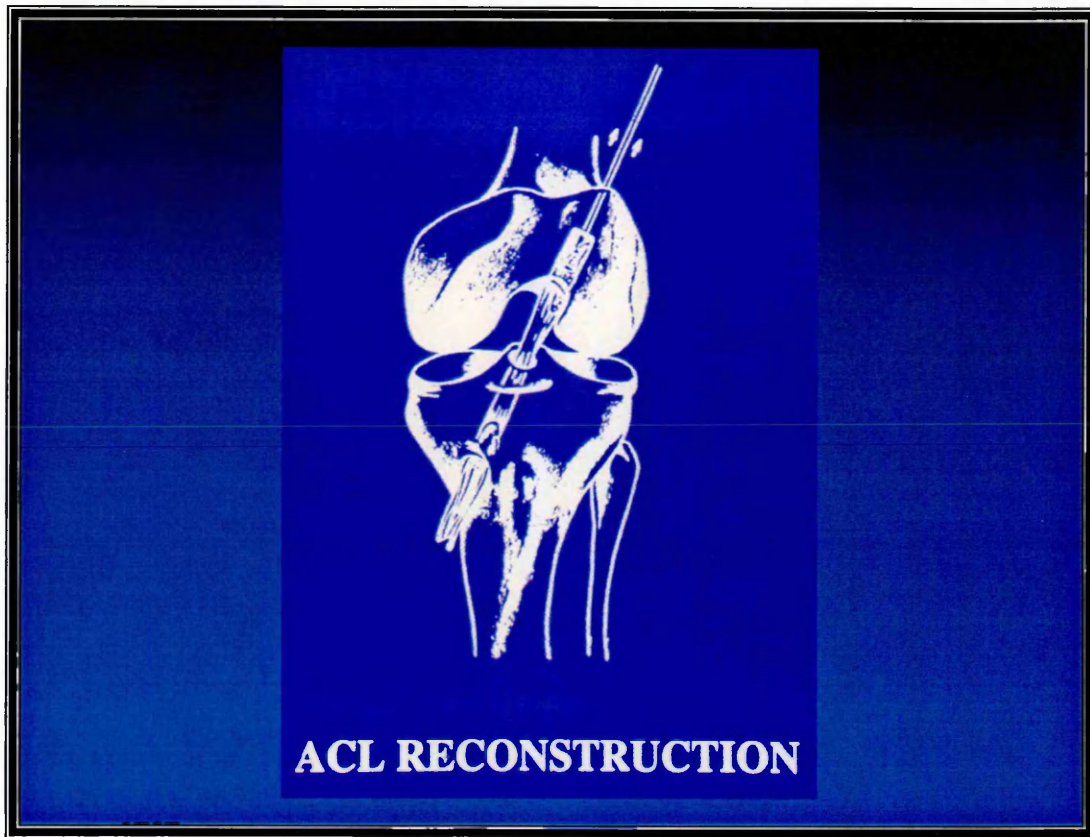


Fig 22. An image of Anterior cruciate ligament reconstruction

At present, there are no validated or standardised decontamination procedures for tissue banks to adhere to, and so each tissue bank has adopted their own procedures. At the LSTS, tendons were initially exposed to 100% ethanol for 10 min to decontaminate them, but this procedure is ineffective as up to 50% of the tissues fail post processing following decontamination and so have to be irradiated. In an attempt to improve the sterility pass rate, the original ethanol procedure has now been superseded by the use of a broad-spectrum antibiotic cocktail. Tendons are soaked in the antibiotic cocktail for 24h at 4°C, but pass rates still remain around 50%, and the cost of the procedure is significantly higher than the former method. As a result, the LSTS continue to terminally sterilise a substantial number of their tendons to ensure sterility.

Studies have shown that gamma irradiation and ethylene oxide can affect mechanical or chemical structure of the tendons and these have been implicated in post implantation failure (Jackson *et al.*, 1988, 1990, 1993; Silvaggio *et al.* 1993, Sterling *et al.* 1995; Smith *et al.* 1996; Toritsuka *et al.* 1997). Several authors have shown that tendons that have been exposed to EtOx contain low levels of the sterilant and high levels of toxic residues: ethylene glycol and ethylene chlorydrin which are formed when EtOx is exposed to water or chlorine, respectively (Paulos *et al* 1988, Jackson *et al.* 1988, 1990). Transplantation using grafts exposed to EtOx have led to adverse reactions such as synovitis and failure of incorporation of grafts. In such cases, the grafts were removed and the synovitis resolved (Paulos *et al.* 1988, Jackson *et al* 1990, McCulloch & Eastlund 1991, Silvagio *et al.* 1991). Due to these adverse reactions, it has been recommended that EtOx sterilised tissue should not be used for ACL or PCL reconstruction until more is learned about the affect of sterilant on tissues (Paulos *et al.*1988).

Investigations have also shown that doses of gamma irradiation produced deleterious effects on the material properties of the graft, resulting in reduced mechanical strength, a property that is required for the success of the implant (Gibbons *et al.* 1989, 1991, Goertzen 1995, Salephour *et al.* 1995, Fideler *et al.* 1995). The allograft acts as a prosthesis and allows the motion of the knee and so the mechanical strength of the tissue is essential for the recovery of the knee.

The surgical demand for tendons continues to rise, especially for tendons without terminal sterilisation to minimise post implantation failure. At present, tissue banks only provide a limited number of tendons without terminal sterilisation and in order to increase the stock, the frequency of terminally sterilised tissue needs to decrease. This can only be achieved if the initial decontamination procedure is effective.

4.2 Material and methods

4.2.1 Preparation of tissue for decontamination treatment

Achilles tendons without muscle tissue of approximately 12 cm² in size were cut in the laminar flow cabinet and then divided into two groups. **Group 1** was spiked with 0.1 ml of panel 1 cocktail that contained 2x10⁴ CFU of *P. aeruginosa*, *E. coli* and *E. faecalis*. **Group 2** was spiked with panel 2 cocktail that contained 10⁴ CFU of *S. saprophyticus*, methicillin resistant *S. aureus*, and *M. luteus*. A further **Group 3** of tendon pieces of equal size **with** muscle tissue attached was prepared. Half of this group (**3a**) was spiked with panel 1 cocktail and the other group with panel 2 cocktail (**3b**).

4.2.2. Treatments

4.2.2.1. **Group 1** samples were divided into 2 groups: **Group 1a** was pre-washed in 0.2% Triton for 15 min and then shaken in 70% ethanol for 15 min at room temperature and **Group 1b** was pre-washed and shaken in undiluted (100%) ethanol for 65 min, at room temperature

4.2.2.2 **Group 2** samples were divided into 2 groups: **Group 2a** was shaken in 70% ethanol for 65 min without a pre-wash at room temperature and **group 2b** was pre-washed before exposure to 70% ethanol for 65 min at room temperature.

All **Group 3** samples were pre-washed in 0.2% Triton for 15 min and then shaken in 70% ethanol for 65 min.

The individual pieces of tissue were then retrieved from the ethanol solutions, dried in air on either side for 10 min, and incubated in nutrient broth overnight. Turbid broth cultures were subcultured on selective media. Spiked tendons without a pre-wash and ethanol wash were cultured overnight in nutrient broth as a positive control. All turbid broths yielded bacterial growth following subculture. Broths that were not turbid, did not yield growth following subculturing.

4.2.3 Statistical analysis

A paired T-Test was carried out for pairwise comparisons between the percentages of tissue found contaminated in each of the treatment groups. A p value ≤ 0.05 indicated significant statistical difference. Advice on statistical methods was given by the Statistics Dept, Centre for Infections, Colindale.

4.3 Results

The strategy for this experiment was to contaminate tissues with two panels of microorganisms as set out in section 2.3, and attempt to reduce their numbers with the aid of ethanol by various different techniques and to see which technique was most effective at decontaminating tendons. Both panels 1 and 2 were not given equal decontamination treatments as there were limited number of tissue available and so it was decided to compare a particular type of treatment within a panel. 70% ethanol treatment was compared with 100% ethanol treatment (1a vs 1b), pre-wash with 70% ethanol treatment was compared with no-prewash with 70% ethanol treatment (2a vs 2b), and 70% ethanol treatment on tendons muscle tissue still attached was compared with tendons without any muscle tissue (1b vs 3a, 2b vs 3b).

The percentages of tissue found contaminated following ethanol treatment in each group are presented in Table 15.

Table 15. The percentage of tissue found to be contaminated following ethanol wash:

	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>E. faecalis</i>
Group 1a (Pre-washed in 0.2% Triton for 15 min, and then shaken in 100% ethanol for 15 min)	50% (12 of 24)	38% (9 of 24)	63% (15 of 24)
Group 1b (Pre-washed in 0.2% Triton for 15 min, and then shaken in 70% ethanol for 65 min)	4% (1 of 24)	0% (0 of 24)	4% (1 of 24)
Group 3a (Muscle tissue still attached, pre-washed in 0.2% Triton for 15 min, and then shaken in 70% ethanol for 65 min)	100% (20 of 20)	100% (20 of 20)	100% (20 of 20)
	<i>M. luteus</i>	MRSA	<i>S. saprophyticus</i>
Group 2a (No pre-wash, shaken in 70% ethanol for 65 min)	8% (1 of 12)	25% (3 of 12)	8% (1 of 12)
Group 2b (Pre-washed for 15 min in 0.2% Triton, shaken in 70% ethanol for 65 min)	0% (0 of 15)	0% (0 of 15)	0% (0 of 15)
Group 3b (Muscle tissue still attached, pre-washed in 0.2% Triton for 15 min, and then shaken in 70% ethanol for 65 min)	100% (20 of 20)	100% (20 of 20)	100% (20 of 20)

Both panels 1 and 2 were not given equal treatments as there were limited tissue available and so it was decided that different type of treatments within each panel were to be looked at.

The results show that as much as 50% in group 1a samples remained contaminated with *P. aeruginosa* and *E. faecalis* following treatment with 70% ethanol for 15 min after a pre-wash. In contrast, as little as 4% of 1b samples (100% ethanol, 65 min) were still contaminated with *P. aeruginosa* and *E. faecalis* following treatment. The percentage of tissue samples found to be contaminated in group 1a is significantly higher than in group 1b, demonstrating that washing contaminated tissue in 70% ethanol for 65 min is more effective than 15 min.

All of group 2b samples yielded no growth after the treatment (n=15) suggesting that sterility can be achieved if an appropriate decontamination procedure is used. Up to 25% of group 2a samples were still contaminated with *M. luteus*, MRSA and *S. saprophyticus* despite being exposed to 70% ethanol for 65 min. This particular group of tissue had not been pre-washed with 0.2% Triton and so the results suggest that tissue should be pre-washed prior to the ethanol treatment.

All of groups 3a and 3b tissue samples (n=20) were still contaminated after treatment. Each of the six different species within the cocktail were recovered from the 48 h broth culture despite being exposed to the ethanol for 65 min after a 15 min pre-wash. The difference between this group and group 1a, 1b, 2a and 2b was that the muscle tissue had not been excised prior to decontamination treatment.

The data in Table 15 shows that ethanol has different effects on the various species. In group 1b, no *E. coli* colonies were recovered from the tendons after treatment, whereas, *P. aeruginosa* and *E. faecalis* were recovered from 4% of the samples. A similar pattern can also be seen in group 1a suggesting that *E. coli* is more susceptible to ethanol than the other two species. Group 2a results also show a difference in species susceptibility to ethanol. Both *M. luteus* and *S. saprophyticus* were recovered from only 8% of group 2a samples, whereas up to 25% of the samples were still contaminated with MRSA.

4.4 Discussion and conclusion

Tissue banks that process tendons have used either ethanol or antimicrobial solution for decontamination prior to terminal sterilisation. Protocols followed by tissue banks vary considerably from one tissue bank to the other. There seems to be no scientific knowledge on how procedures work, the efficiency of the procedure or if there are any factors that may interfere with the antimicrobial activity. For the first time, this study has validated a decontamination procedure and has shown factors that interfere with the process.

4.4.1 Ethanol concentration and exposure time

Ethanol is only able to kill vegetative forms of bacteria rapidly provided sufficient water is present to give a final alcohol content in the reaction mixture of between 60 and 70% (Whittet *et al.* 1965). The original treatment that was carried out at LSTS using 100% ethanol was therefore practically useless against microorganisms, which explains why only a low number of tendons passed the sterility test. In fact, 22% of 50% tendons that did pass the sterility test were sterile prior to decontamination. Before embarking on any kind of tissue decontamination, especially against bacteria using alcohol, it is vital that alcohol is in an aqueous state, ideally 70%.

Another factor that plays an important role in the antimicrobial activity of ethanol is exposure time. However, the exposure times to kill various bacteria such as *E. coli* and *S. aureus* do depend on alcohol concentration. Examples of exposure time and concentration required to kill various types of bacteria can be found in McCulloch (1946). The susceptibility of organisms to the antimicrobial activity of ethanol varies with the species (Whittet *et al.* 1965, McCulloch 1946). At 70%, it requires 60 min to

kill both *E. coli* and *S. aureus*, whereas at 80%, for *E. coli* and *S. aureus* 24 h and longer 3 than days, respectively is required (McCulloch 1946).

4.4.2 Factors affecting the anti-microbial activity of alcohol

It is known that alcohol based products are not recommended for spillages containing organic matter. Organic matter in the form of serum, blood or pus are thought to interfere with the antimicrobial activity of the alcohol in two ways (Ratula 1997).

Chemical reaction between the alcohol and the organic matter occurs resulting in a complex that is less germicidal.

- 1) When organic matter comes in contact with alcohol, it coagulates around the bacteria that may be present, so protecting them from desiccation.
- 2) When organic matter comes in contact with alcohol, it coagulates around the bacteria that may be present, and so protecting them from dessication.

Since desiccation is a major factor in the death of viable cells (Kligman 1965), it would be necessary to expose all cells to ethanol, taking note that different bacteria differ in susceptibility to drying (McCulloch 1946, Marples 1965, Whittet *et al.* 1965). All of group 3 tendons were found contaminated following ethanol treatment. These tendons still had their muscle tissue attached during the treatment and so contained sufficient amounts of blood to trap viable cells during coagulation, which protected the different species against desiccation.

Species such as *M. luteus* and *S. saprophyticus* can occur as microclusters (Kligman 1965, Marples 1965, Williamson & Kligman 1965). To achieve complete death of all viable cells with ethanol, it would be necessary to break up these clusters, so that the protected cells are exposed to the ethanol. A mild detergent such as Triton or Tween X100 breaks up microcolonies or clusters quite effectively (Williamson & Kligman

1965). The benefits of a wash with a mild detergent can be seen when comparing the results between group 2a and 2b (Table 15). All of group 2b tendons that were pre-washed with Triton were found sterile, whereas, as many as 25% of group 2a samples were still contaminated despite being washed in 70% ethanol for 65 min. Washing tendons with 70% ethanol has been proven to be effective at killing bacteria, however it must be noted that ethanol is active only against vegetative forms of bacteria, but not spores (Russell 1990, Whittet *et al.* 1965), and so spore formers such as *Bacillus* spp. will survive the ethanol treatment. Ethanol cannot be regarded as a sterilising agent as it is only active against surface contaminants. Any contamination within the cortex of the bone will survive. However, ethanol does possess both bactericidal and virucidal properties and appears to be effective against HIV (Morgan *et al.* 1993). Ethanol is also useful for eradication of fat and marrow elements from larger allograft segments. Effective donor screening and prompt tissue retrieval following death of the donor will minimise any endogenous tissue contamination. To ensure that there is no endogenous contamination, it is recommended that bone chips from the tendon bone block are sampled for bacterial contamination at pre and post processing. It is also recommended that tendons should be terminally sterilised if bacteria are recovered from bone chips or *Bacillus* species from the surface of the tendon before or after processing.

Studies have been carried out using ethanol to decontaminate bone specimens (Dahners & Hoyle *et al.* 1989, Hooe & Steinberg 1996), and demonstrated that immersing contaminated bone in 70% for 8 h achieved complete sterilisation. However, both groups have also demonstrated that ethanol can have deleterious effects such as decreased osteoinductive properties of the bone and bony necrosis. Tendons treated with ethanol become dry and brittle, however when washed with saline several times, regain hydration and flexibility. Nevertheless, it is not known if ethanol has any

deleterious affects on the biochemical or mechanical strength of tendons rehydrated after treatment.

Chapter 5

Recommendations

Strict standards must be applied to avoid disease transmission from donor to recipient involving donor medical history, donor testing for viral diseases, aseptic retrieval and processing, and control of storage temperature. During aseptic processing, precautions must be taken to minimize the introduction of new organisms. Bioburden assessment and processing of tissue should be carried out using sterile equipment, in a clean room with positive pressure, inside a class II cabinet and staff wearing protective gowns, caps and face masks.

Staff should be educated on the basic routes of contamination, how and why aseptic techniques are applied during retrieval, processing and packaging of tissue. They should be instructed on how disinfectants work, and have an understanding of the microbiological aspects of the methods used for processing tissue such as using the correct techniques for sampling tissue.

5.1 Tissue retrieval:

1. Since ventilation systems within mortuaries vary considerably from poor to non-existent, and the type of work carried out in the mortuary, it is important that all necessary preventative measures are taken to prevent staff contaminating tissue through bacterial shedding.
2. Retrieval should ideally not be carried out in mortuaries due to the high rates of contamination with pathogenic organisms, and the unreliability of testing methods for bacteriological load. Secondary sterilisation should be carried out on all tissues retrieved in mortuaries.

3. Even though no investigations were carried out on the type of skin disinfectant. This study does suggest that the 0.3% chlorhexidine gluconate, in 70% industrial methylated spirit may not be the ideal product for donor's skin disinfection since organisms such as *E.coli* were still recovered from tissue. The use of isopropyl alcohol followed by iodine tincture is thought to be more effective as a skin disinfectant than the combination of chlorhexidine gluconate and isopropyl alcohol and it is suggested that this needs to be looked at as an alternative product.
4. Instruments should be covered at all times during donor preparation or when other activities are taking place within in the mortuary as shown in case 14, where an identical strain of *Micrococcus* spp. was recovered from tissue, staff and settle plates placed on the instrument field, and in case 19, an identical strain of *S. epidermidis* was only recovered from settle plates and staff.
5. To minimize the bacterial load, retrievals should ideally be carried out in operating rooms, using aseptic techniques with only a few personnel for procurement. However, this is clearly not practical and therefore care must be taken to reduce the risk factors associated with retrievals in mortuaries.

6. To avoid cross contamination, each type of graft should be packed separately. Ideally, gloves should be changed between each tissue removal.
7. The interval between postmortem time and retrieval should be kept to a minimum.

5.2 Bioburden assessment:

1. It is recommended that swab culturing should not be used to assess relatively low levels of microbial contamination on allografts. Culturing the filtered wash of bone will detect both low level and high levels of contamination as this has proven to be a sensitive, quantitative and qualitative technique even though not reproducible.
2. If a swab culturing technique is performed, it is vital that a moist swab is used as it is more effective at sampling. The swab should be placed into a selection of enrichment broths that will allow growth of all types of bacteria. Swabbing straight onto dry medium is not effective.
3. The swabbing technique needs to be addressed. Thorough sampling of the surface area of the graft is vital to ensure detection of low level contamination.

5.3 Decontamination procedures:

1. Tissue bankers must employ an effective decontamination procedures

during processing to ensure that all types of bacterial contamination are eliminated.

2. To achieve sterilisation of tendons with ethanol (providing that tendons are not contaminated with spores, and spore forming bacteria):
 - i. as much as possible muscle tissue must be removed from the tendon.
 - ii. the tendon must be pre-washed with a mild detergent to break up bacterial clusters.
 - iii. the ethanol must be in an aqueous state, at least 70% to be an effective sterilising agent.
 - iv. the tissue must be kept in ethanol for at least 60 min to allow protein coagulation and hence death of cells.

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7. Appendices

7.1 Appendix 1. Ingredients

7.1.1. Bacterial recovery solution:

Dissolve a phosphate buffered saline (PBS) tablet in 100 ml of purified distilled water.

Warm up PBS solution to about 40°C to allow the Triton to dissolve efficiently.

10% Triton: 10 ml of Triton in 90 ml of PBS

0.1% Triton: 1 ml of Triton in 99 ml of PBS

The pH of the recovery solution should be 7.3

Aliquot 2 ml of the solution into sterile bijoux and autoclave at 130°C for 15 min.

During sterilisation, the Triton tends to settle to the bottom and so, while bottles are still hot, the bottles are shaken to re-dissolve the detergent.

7.1.2. Agarose Gel

0.9 g of Certified Molecular Biology Agarose (Bio-Rad Laboratories, UK)

6 ml of 5X TBE Buffer (made from 10X TBE Buffer (Tris, Boric acid, EDTA),
Invitrogen, UK).

54 ml of distilled water.

Make up the solution, add the agar and heat in microwave or steam bath until fully dissolved. Cool to about 60°C and pour into gel tray with comb. Allow to set before running products.

7.2. Pilot studies

7.2.1. Effects of dehydration on organisms

Whyte and Niven (1986) showed that the number of colony forming units (CFUs) are reduced by dehydration, and the death rate varies from species to species. For example, *S. aureus* survived well compared to *E. coli* in a defined time of dehydration. Spiking of tissue involves covering a known area of the allograft tissue with bacterial suspension and a period of time is allowed for adhesion of the bacteria to the tissue to take place. During this period, dehydration of the organisms may take place and hence reduce the viable counts. Allograft tissue spiked with bacteria were used to compare the different recovery processes. A selection of strains were chosen to represent species that are most commonly isolated from tissue during sterility testing. These could be phenotypically distinguished from each other and be isolated specifically by the use of selective media in the recovery process.

Bacterial species and culture conditions

The species were:

E. coli

E. faecalis

P. aeruginosa

Bacillus spp.

M. luteus

Methicillin resistant *aureus*

S. saprophyticus

The strains were inoculated into nutrient broth and incubated at 37°C overnight. The CFU were counted by serial logarithmic dilution. *E. coli* should be approximately 1×10^9 CFU/ml and for *S. aureus* should be 1×10^8 CFU/ml.

Inoculation and drying

Non-woven cellulose acetate (NCA), (J-Cloth) were cut into 50 x 30 mm pieces wrapped in tin foil and autoclaved at 121 °C for 15 min. A set of 18 to 20 pieces of sterile NCA were hung on wires strung within a cardboard box of approximately 30 x 20 cm to protect the cloths from draughts and disturbance in the laboratory for each strain. The cloths in each set were then inoculated with 0.1 ml of bacterial suspension of a single strain in each experiment. The cloths were then allowed to dry in air for different time periods. Two cloths were removed at the end of each drying time and immediately placed into separate 10 ml volume of PBS with 0.1% Triton and vortexed for 2 min. The solution was serially diluted a 100-fold (10^{-2} , 10^{-4} and 10^{-6}) with quarter strength Ringer's solution and 0.1 ml of each dilution was spread on nutrient agar and incubated at 37°C for 48 h before recording the number of colonies.

The period included at time zero (which was defined as the time when the cloth was first inoculated), at 1 h, 2 h, 4 h, 6 h, 24 h and 48 h. Preliminary tests had showed that the time taken for a wet cloth to dry completely took approximately 1 h.

Temperature and humidity in the sampling area could not be controlled, although the temperature was recorded.

Table 16. The effect of dehydration on the survival of species chosen for the cocktail panels.

Time in hours	<i>E. coli</i> Average CFUs in 0.1ml	<i>E. faecalis</i> Average CFUs in 0.1ml	<i>P. aeruginosa</i> Average CFUs in 0.1ml	<i>Bacillus spores</i> Average CFUs in 0.1ml	<i>M. luteus</i> Average CFUs in 0.1ml	<i>MRSA</i> Average CFUs in 0.1ml	<i>S. saprophyticus</i> Average CFUs in 0.1ml
0	7.255	7.579	7.176	6.477	7.491	7.301	7.602
0.5	6	5.477	6.662	7.301	7.255	5.74	5.851
1	5.342	4.477	5.832	6.903	7.255	5.568	5.826
2	5.146	4.698	5.748	7.204	7.255	5.579	5.748
4	4.222	3.477	5.079	6.845	6.903	5.556	5.77
6	3.857	4.276	4.296	7.079	7.079	5.322	5.653
24	3.477	4.12	0	6.845	7.23	4.301	5.204
48	2	3.414	0	6.698	7.23	4	5.278

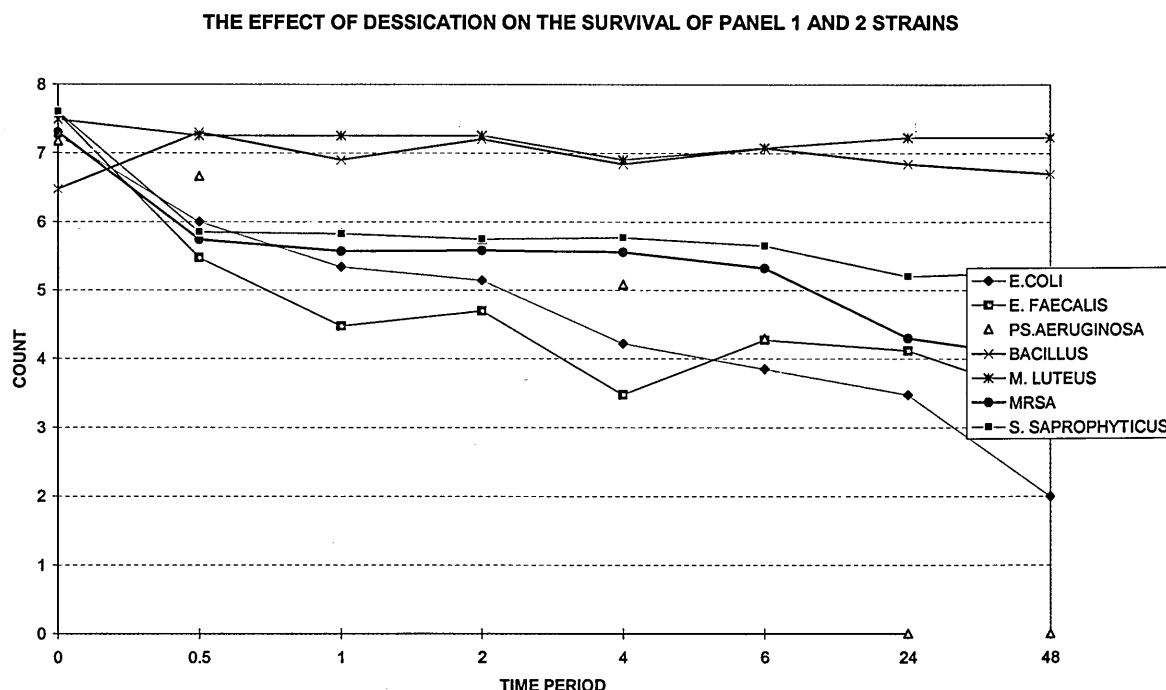


Figure 23. The effect of dehydration on the survival of panel 1 and 2 strains.

7.2.2. The effects of PBS and 0.1% Triton and refrigeration on viable cells over a 72 h time period.

Owing to the sometimes delay of culturing specimens taken out of regular work hours, it was necessary to confirm that suspending solutions for the specimen were not deleterious for the bacteria being recovered. An experiment was designed to assess the effects of PBS and 0.1% Triton on viable cells over a 72 h time period in refrigeration condition.

A 10 ml aliquot of staff cylindrical skin scrub was held 4°C and 0.1 ml aliquot of the sample were subcultured in day 1, at 24 h, 48 h and 72h. The viable counts were determined by standard methods.

Results:

Day 1: average recovered 778 CFUs

24h: average recovered 543 CFUs

48h: average recovered 385 CFUs

72h: average recovered 158 CFUs

7.2.3. Are all the CFUs recovered from the swab?

Overnight cultures of panel 1 strains were prepared as a cocktail (2:2:1 ratio) and then 10 fold serially diluted using 9 ml 1/4 strength Ringers.

A control was set up by diluting 10^{-4} to 10^{-6} and diluting 10^{-6} to 10^{-7} with 9.9 ml PBS and 0.1% Triton.

Test:

1. 0.1 ml of 10^{-4} bacterial suspension was allowed to absorb into a cotton swab.

The swab was then placed in 9.9 ml of 0.1% Triton.

2. 0.1 ml of 10^{-5} dilution was absorbed in a cotton swab.

Both step 1 and 2 were repeated.

Results:

Colony count:

Organism	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
<i>E. faecalis</i>	HG	319	25	2
<i>P. aeruginosa</i>	HG	227	50	6
<i>E. coli</i>	HG	173	18	3

10⁻⁶

	<i>E. faecalis</i>	<i>P. aeruginosa</i>	<i>E. coli:</i>
1	8	1	7
2	19	17	8
3	14	14	9
4	10	13	4
5	26	13	13
Average	15.4	11.6	41

The percentages recovered of the original inoculum (10⁻⁶) on the swab were as follows:

Species	Percentage recovered from the swab
<i>E. faecalis</i>	61
<i>P. aeruginosa</i>	23
<i>E. coli</i>	45

10⁻⁷

	<i>E. faecalis</i>	<i>P. aeruginosa</i>	<i>E. coli:</i>
1	2	0	0
2	0	2	0
3	4	2	3
4	0	2	4
5	2	1	3
Average:	1.6	1.4	2

The percentages recovered of the original inoculum (10⁻⁷) on the swab were as follows:

Species	Percentage recovered from the swab
<i>E. faecalis</i>	80
<i>P. aeruginosa</i>	23
<i>E. coli</i>	66

Overall % recovery for all strains: *E. faecalis* (71%), *P. aeruginosa* (23%), and *E. coli* (56%).

7.3 **Appendix 2. RAPD analysis**

7.3.1. **Generating a dendrogram from RAPD patterns.**

1. The RAPD image is scanned into the program database, distorted bands are straightened and unwrapped to produce a version of the gel, with the help of image-processing software.

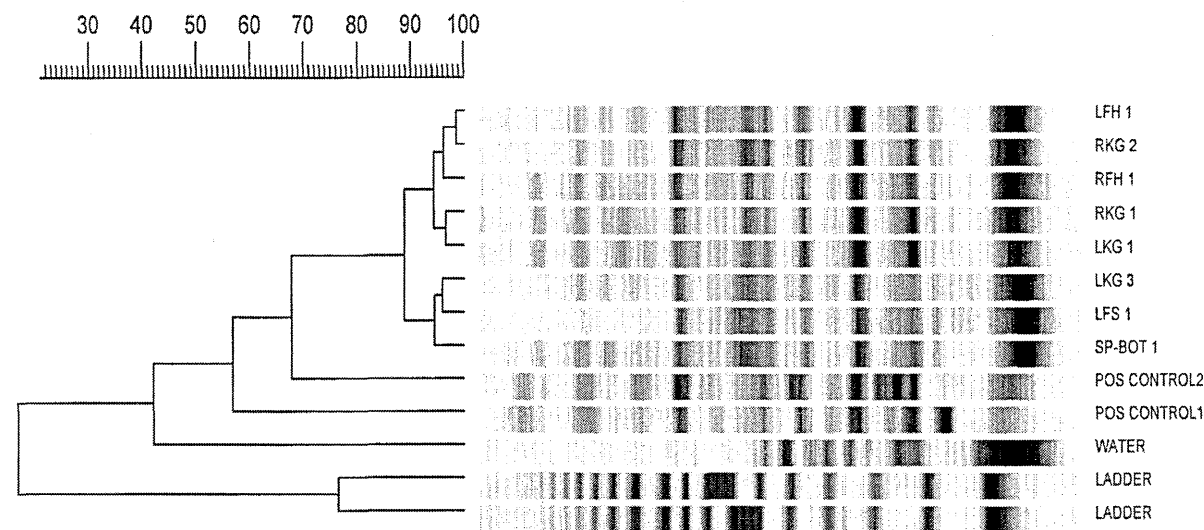
2. The image of the processed gel is analysed automatically, where the program identifies each lane and band; the pixel density of each band is then converted into classes of reflecting intensity.
3. RAPD patterns are compared by their molecular weight and band intensities and logged in a text map, or densitometry map.
4. The program generates a dendrogram using data in a text map while computing similarity coefficients between pairs of isolates. In the generation of the dendrograms, banding patterns of isolates are normalized into a global standard, and in theory different patterns of the same organism can be compared even though they are performed years apart.

Gel Compar automatically processes the gel image, performs image analysis, computes similarity coefficients, generates the dendrogram, and stores the data for future analysis.

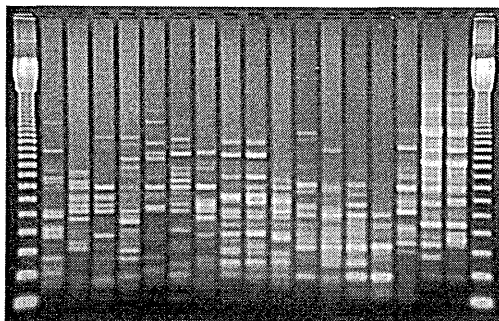
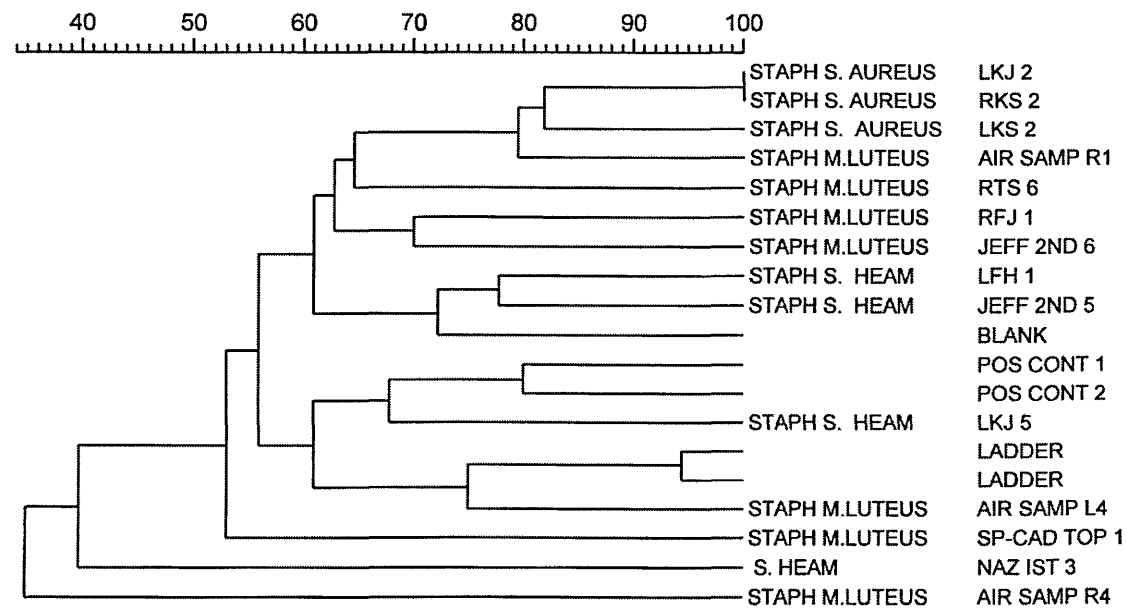
7.4. Appendix 3: Results

7.4.1. Results of RAPDs, dendrogram

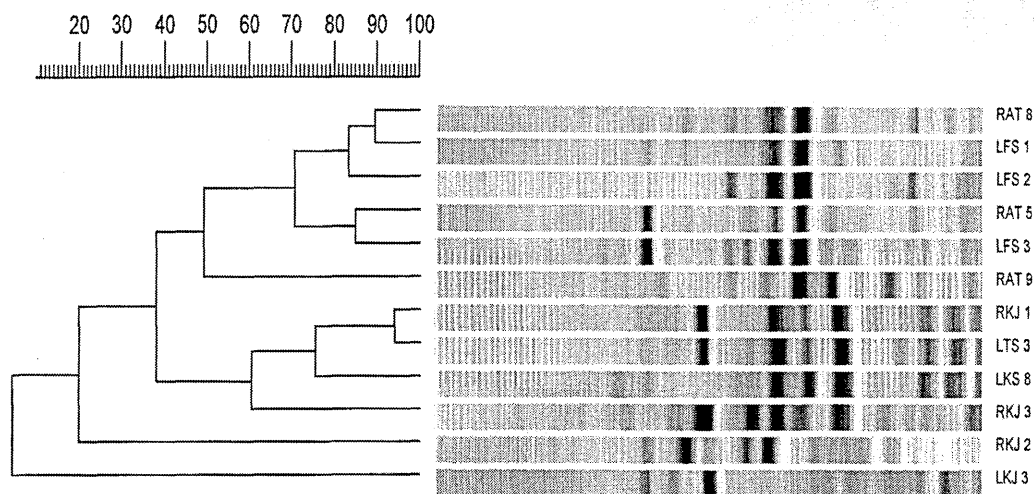
093560w3, *E. coli*



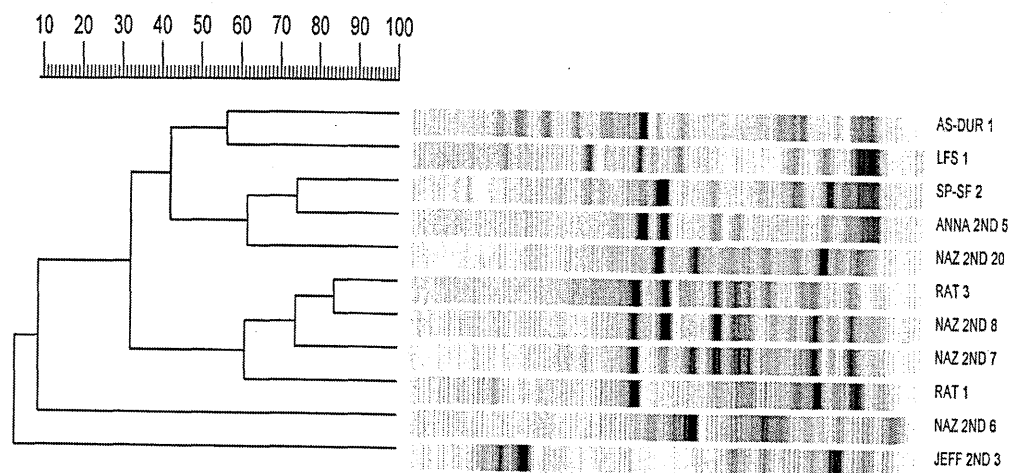
Case 093917wx, *S. aureus*



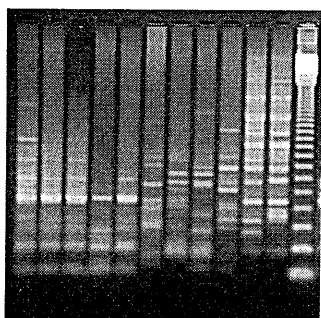
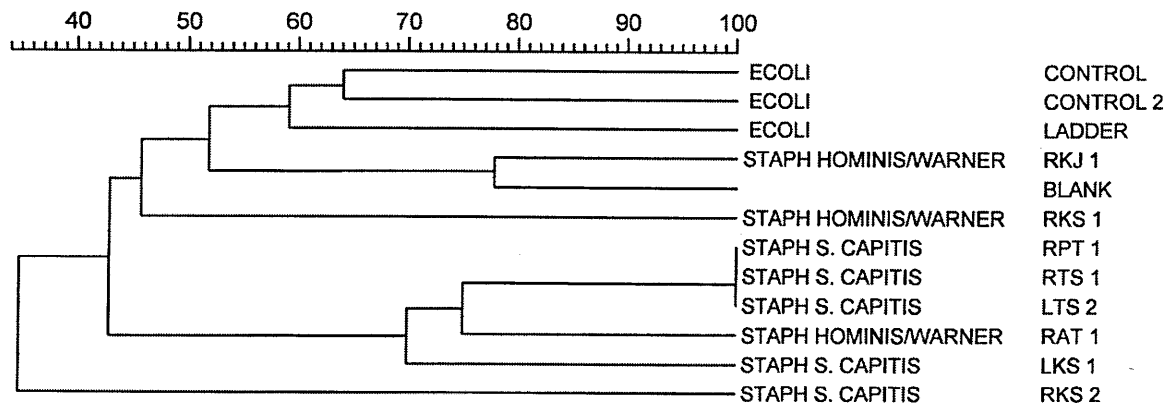
094593w3, *S. capitis*



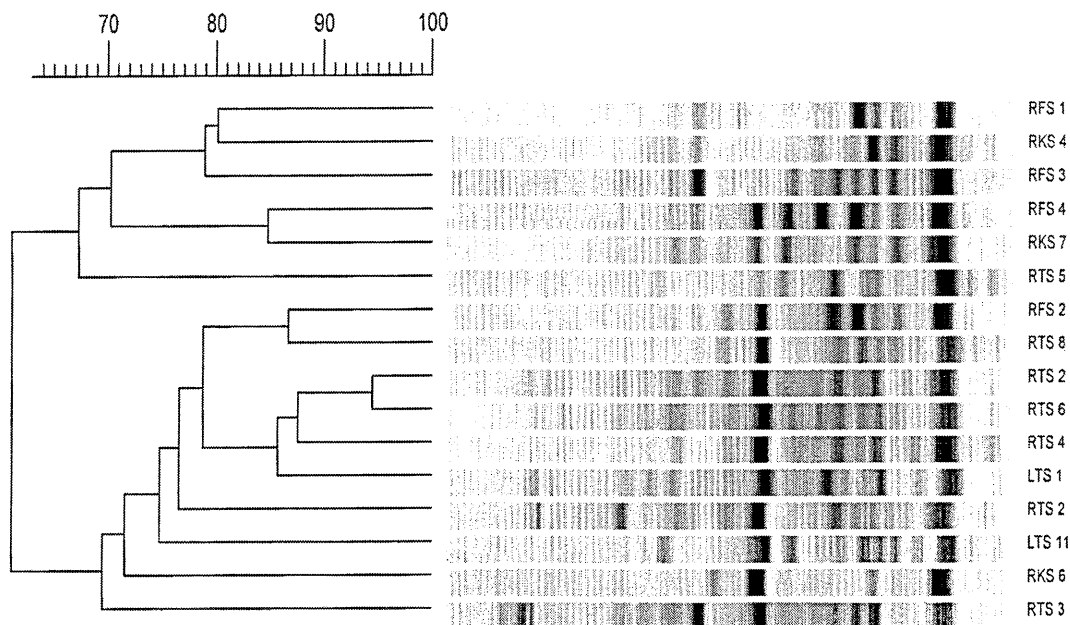
093590w5, *S. warneri*



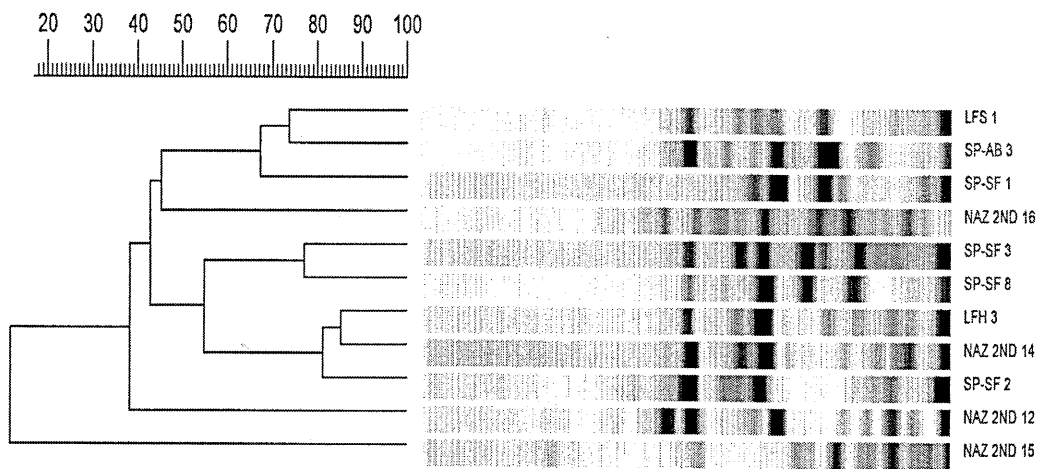
093170w3, *S. capitis*



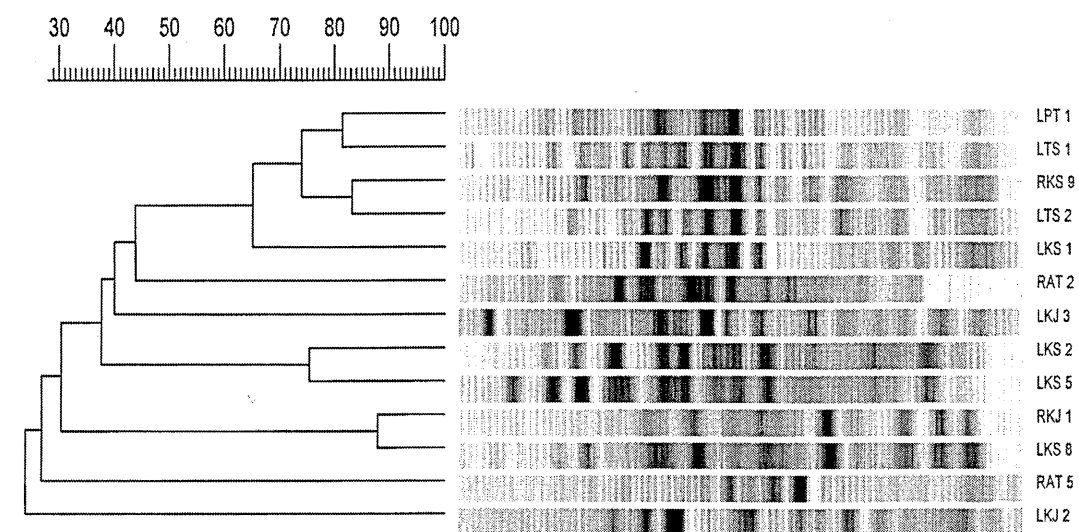
094120w4, *S. haemolyticus*



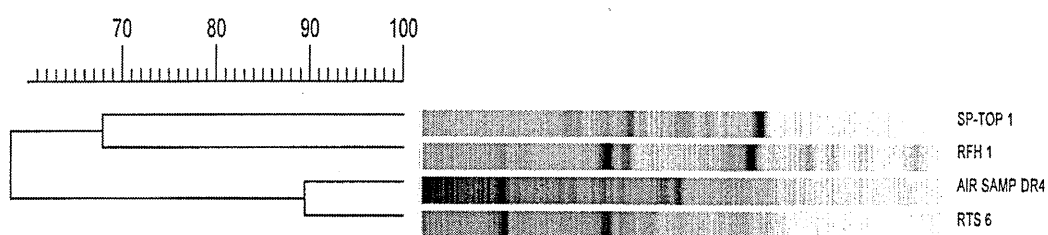
094120w4, *S. epidermidis*



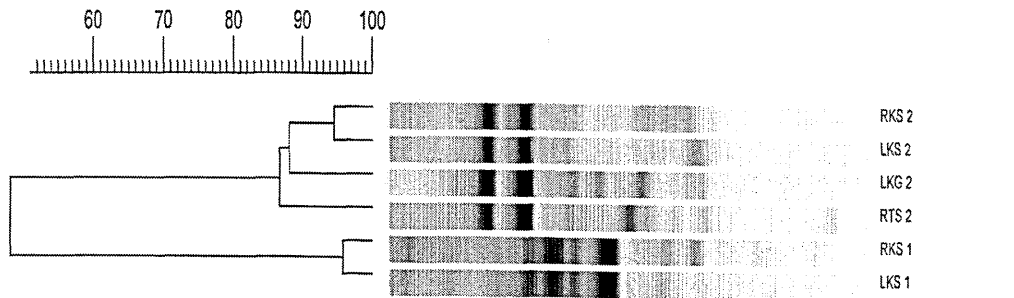
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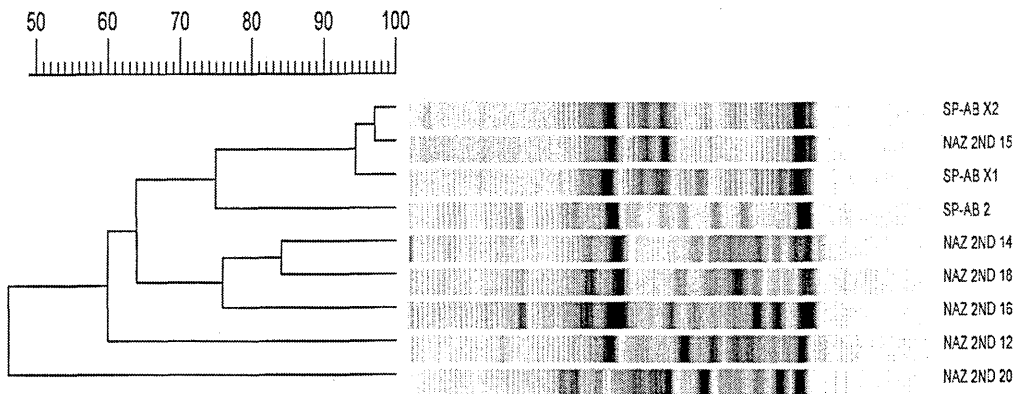
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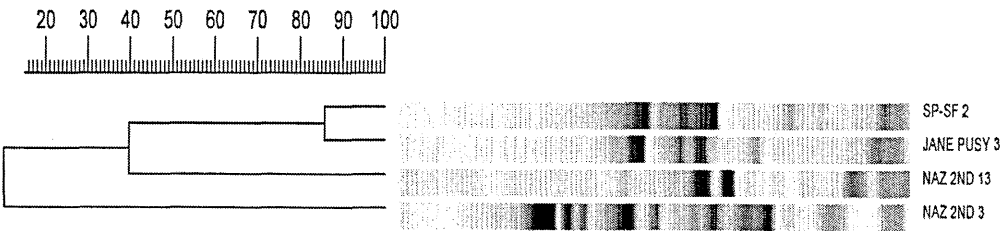
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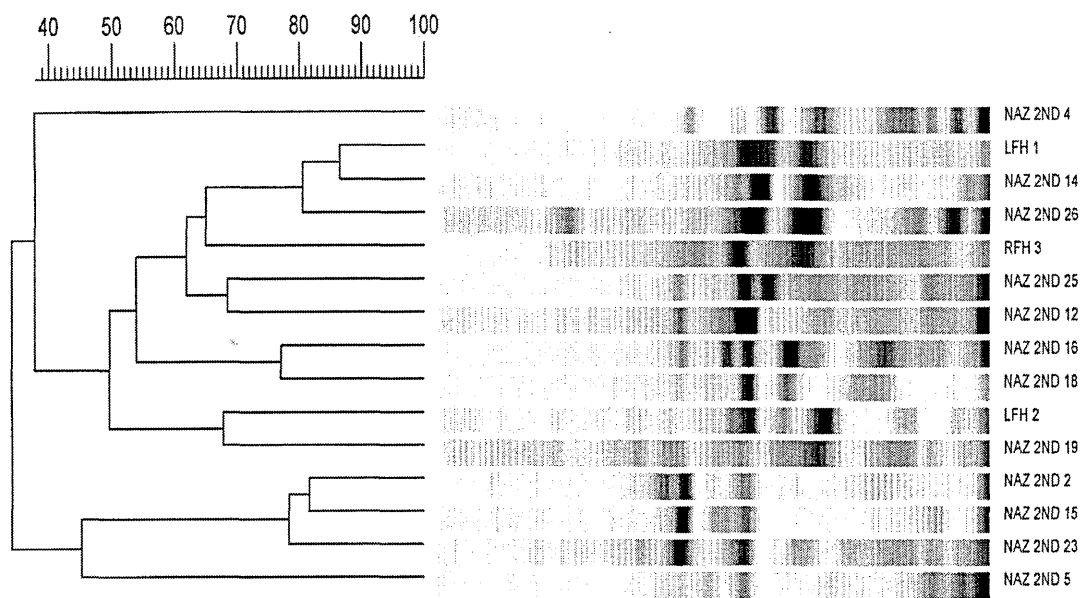
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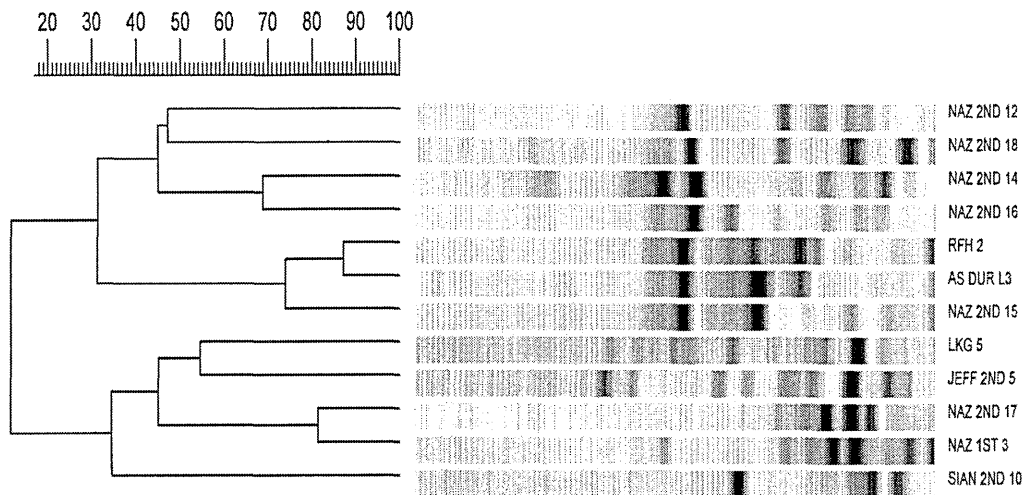
093929w3, *S. capitis*



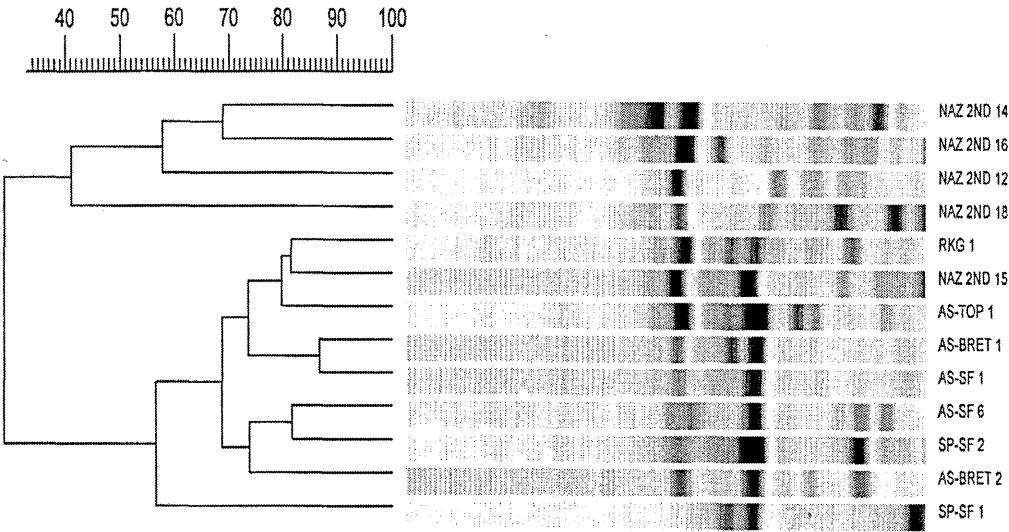
093929w3, *S. saprophyticus*



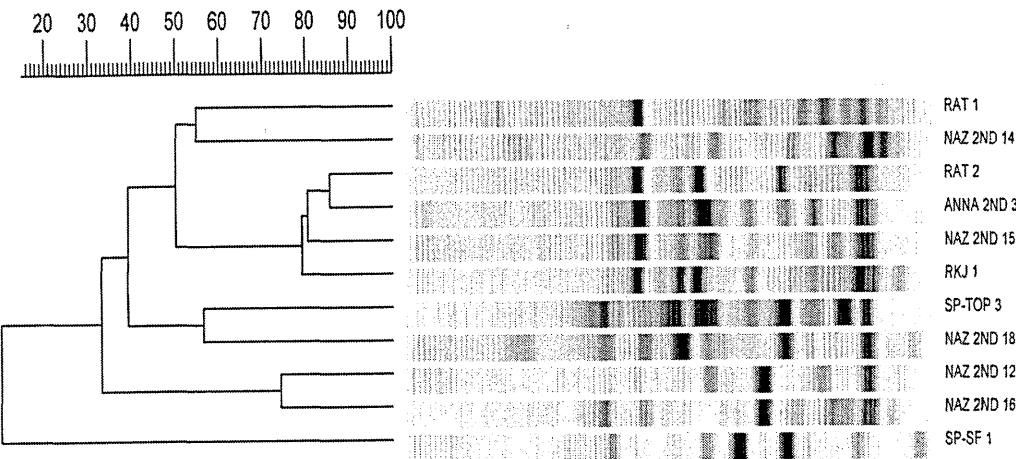
093917wx, *S. epidermidis*



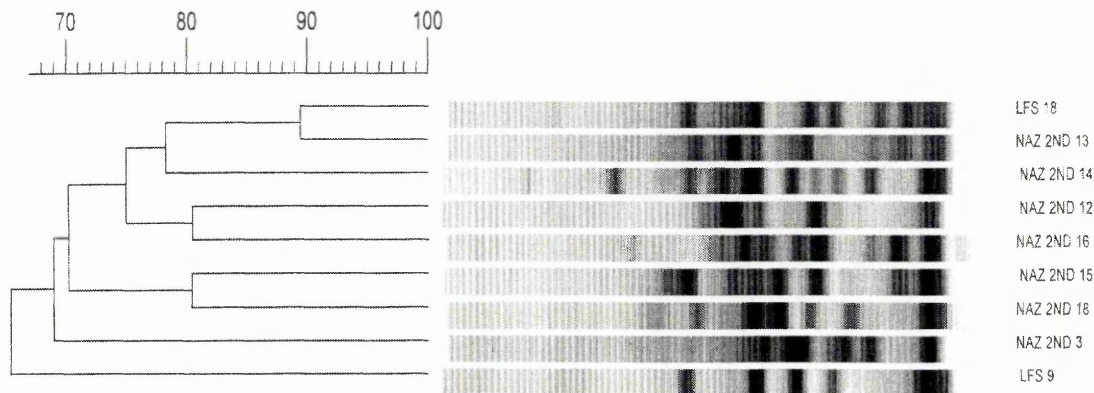
093010w5, *S. epidermidis*



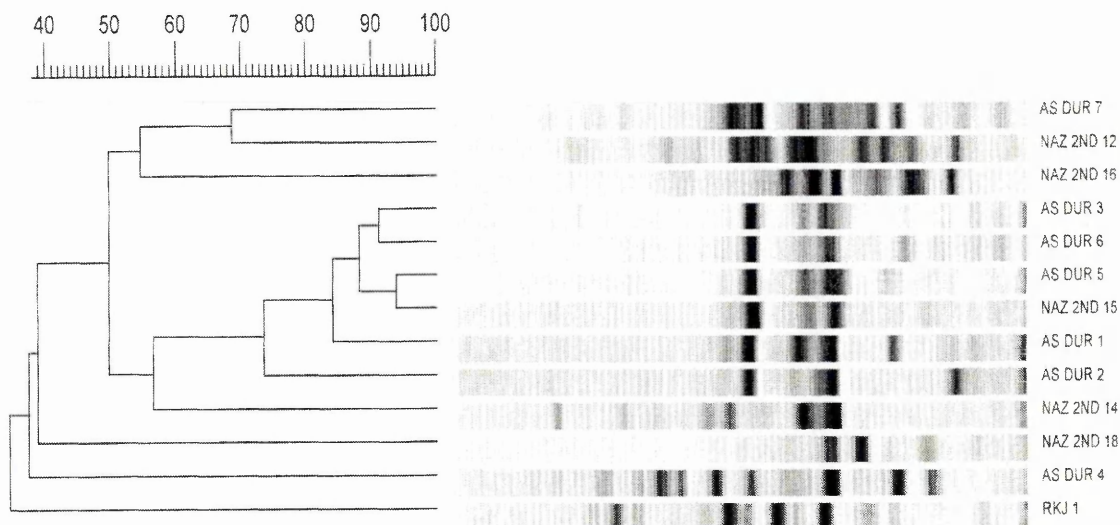
093590w5, *S. epidermidis*



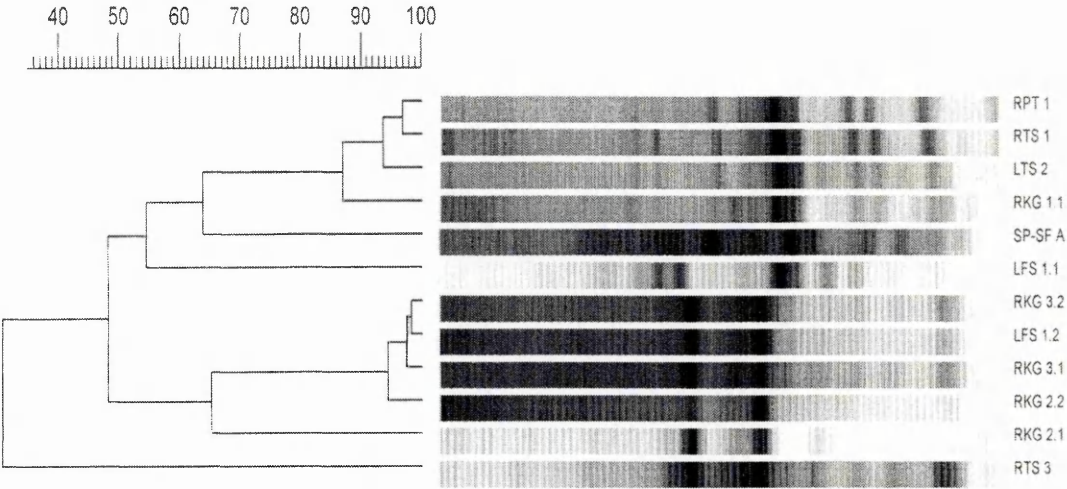
G07579710154N, *S. capitis*



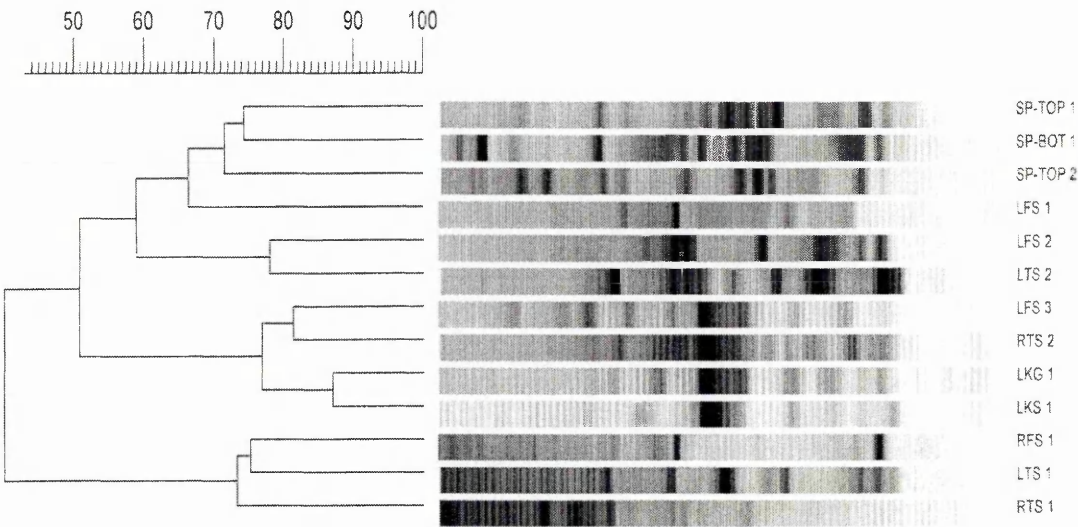
G07579710188, *S. epidermidis*



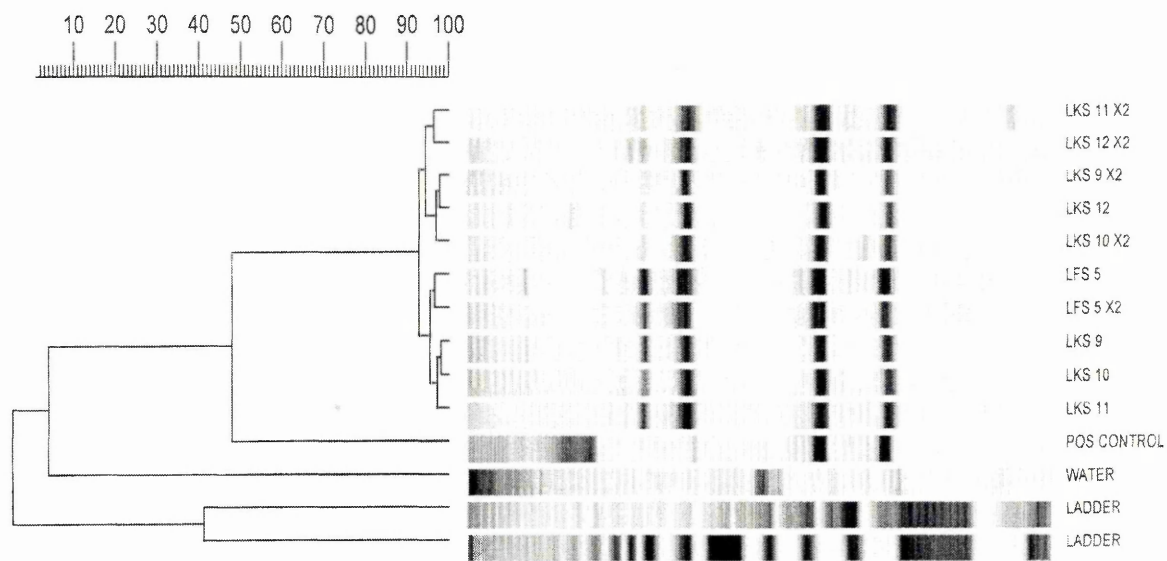
091930w6, *S. epidermidis*



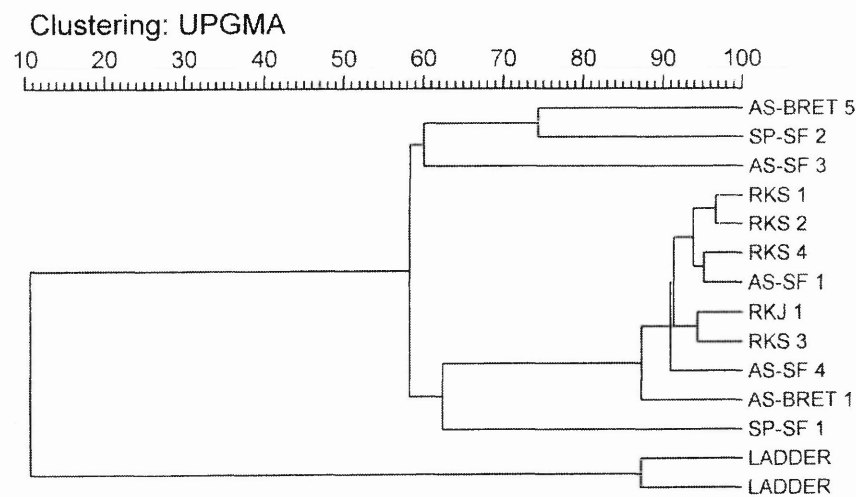
093180w2, *S. epidermidis*



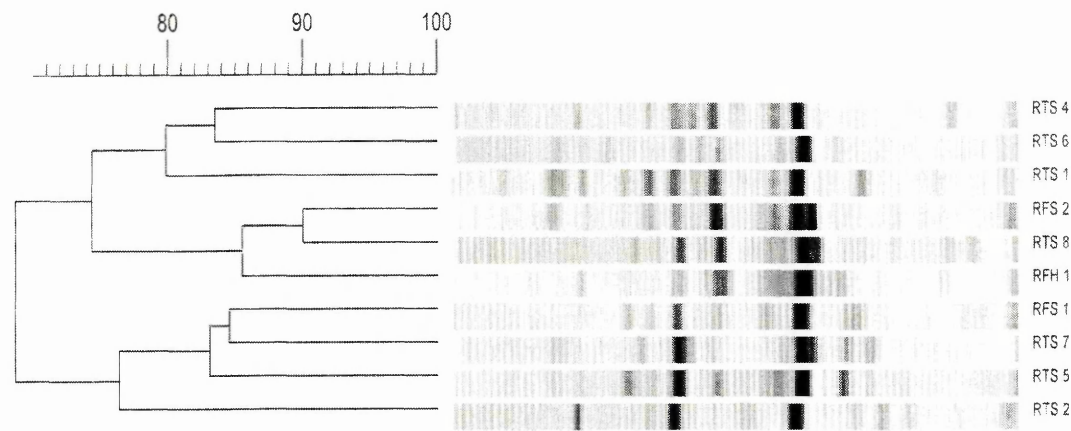
G07579710154N,
E.coli



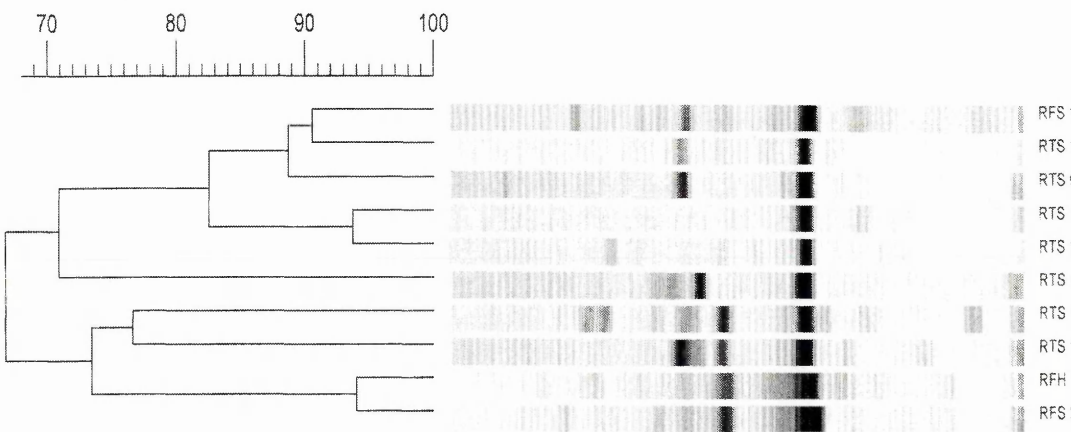
093010w5, *S. epidermidis*



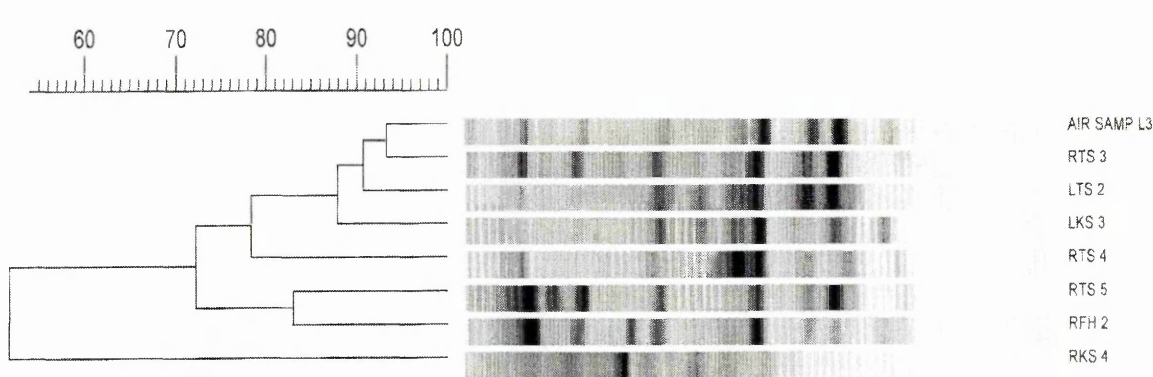
094780w6, *S. lentus*



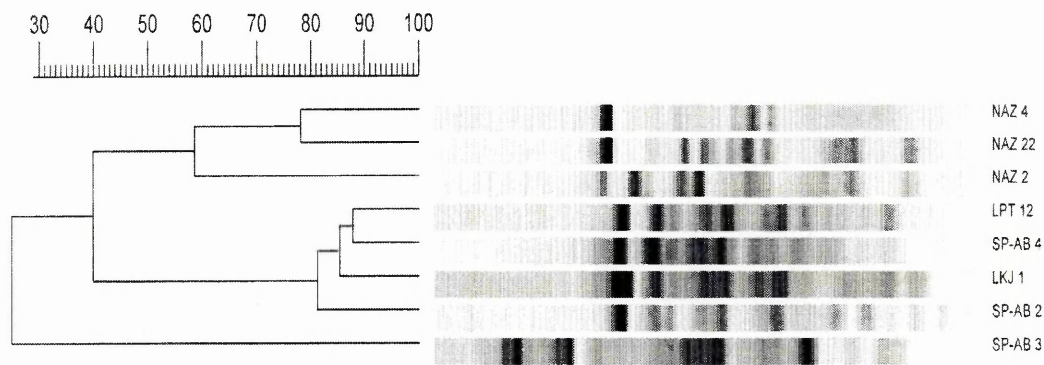
094780w6, *S. lentus*



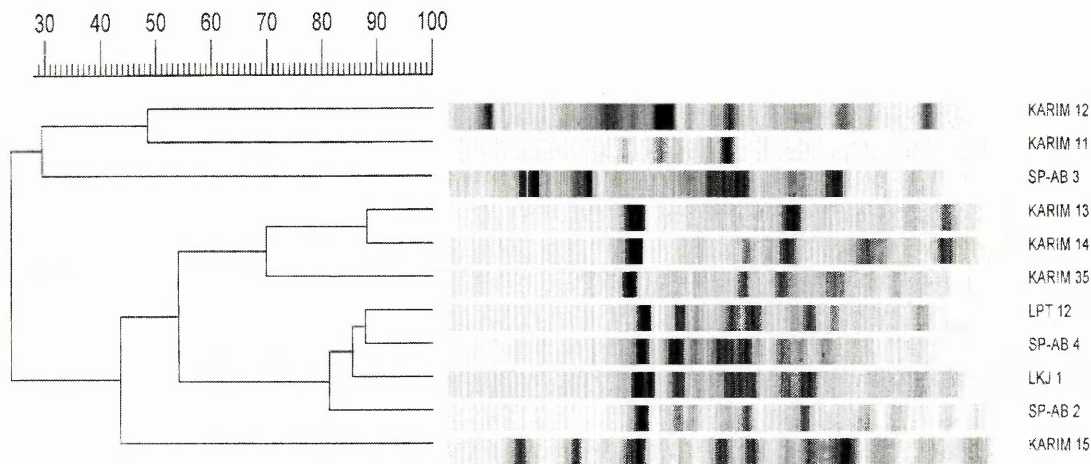
093417wx, *S. epidermidis*



094538w2, Micrococci



094538w2, Micrococci



094538w2, Micrococci

